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Factors influencing glycogen branching enzyme activity in mouse liver

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FACTORS INFLUENCING GLYCOGEN BRANCHING ENZYME ACTIVITY IN MOUSE LIVER

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ABSTRACT

Glycogen is the storage polysaccharide in a spectrum of organisms ranging from yeast to humans. Glycogen has a highly branched structure somewhat resembling a tree or shrub, with the branch points formed by glucose molecules being joined by α 1,6-glycosidic linkages. These linkages are formed by the action of branching enzyme. Branching of glycogen is critical to health, with a lack of branching causing glycogen storage disease type IV, which is fatal in humans by age 2.

While glycogen branching enzyme has been studied in clinical medicine, modulation of the enzyme has received comparatively little attention. This dissertation undertook three experiments to address whether or not branching enzyme changes in response to dietary intervention and supplementation in mouse liver.

Experiment one focused on the application and refinement of a quantitative assay technique for the measurement of branching enzyme activity. The experiment tested the reliability of the assay for the first time on a large number (n=68) of subjects. Intraclass correlation coefficients revealed that the technique demonstrated high reliability (ICC's of 0.848, 0.925, 0.99, and 0.998) for the various assay components. A simplification of the assay method was also introduced that increases the economy of the testing protocol.

Experiment two examined whether or not a dietary intervention resulted in different degrees of branching in the liver glycogen of two groups of mice. The results did not show a significant difference between mice fed on low-carbohydrate versus high-carbohydrate diets.

The third experiment investigated whether diet combined with quercetin supplementation had an effect on the degree of branching of mouse liver glycogen. Of the nine cohorts under

study, only two groups were significantly different from one another statistically ($p = .049$). These two groups differed only in the time of day the animals were euthanized. Glycogen concentration was not different between the two groups.

Taken together, the experiments provide data indicating that the assay technique is reliable. New data pertaining to norms in the degree of branching in mouse liver glycogen are reported as well. Future experiments could apply the assay to other tissues such as skeletal muscle in conjunction with exercise.

CHAPTER I – LITERATURE REVIEW

General Introduction

Virtually every organism from yeast to primates stores carbohydrate in the form of glycogen, which can be readily mobilized to meet energy needs during fasting and muscular activity. The French physiologist Claude Bernard first identified a starch-like substance in liver and muscles and named the substance glycogen, or "sugar former," in 1870 (*1*). In the years since this discovery, scientific inquiry into the structure and function of glycogen has led to discoveries that are recognized as landmarks in modern biology.

Glycogen was the first biopolymer to be synthesized in a test tube. The first inborn error of metabolism attributable to the deficiency of a single enzyme is a glycogen storage disease. The mechanism of insulin action has been revealed largely by studies of the control of glycogen synthesis and degradation. Among the broad principles emerging from the study of glycogen include covalent phosphorylation as a control mechanism in metabolism. Covalent phosphorylation was discovered by Carl and Gerty Cori as the mechanism of activation for glycogen phosphorylase. With the discovery of glycogen synthase by Leloir and Cardini, another enzyme in glycogen metabolism was found to be regulated by phosphorylation, in this case inactivated (*2*). Mitochondrial pyruvate dehydrogenase was then found to be controlled by reversible phosphorylation in a fashion similar to that of glycogen synthase. This discovery led to the acknowledgment that control by phosphorylation is a recurring theme in biological systems, going beyond the bounds of glycogen metabolism. The discovery of cyclic AMP and the development of the concept of second messengers emerged due to the investigation of the control of glycogen phosphorylase by epinephrine, which also led to an understanding of

intracellular signaling amplification by the cAMP cascade. Studies on glycogen synthase also led to an understanding of the significance of multiple phosphorylations.

This review will trace the development of some of the key areas of scientific inquiry on glycogen metabolism. The review will focus on the mechanisms of glycogen biosynthesis and degradation by examining the regulatory proteins involved in the building up and breaking down of the glycogen macromolecule. The metabolic pathways of glycogen synthesis and breakdown are relatively short. This will allow for a detailed treatment of the enzymes involved and will provide a useful framework for understanding how glycogen metabolism is controlled. Also, some of the areas of debate and obscurity in the current state of understanding of glycogen metabolism will be revealed.

One area of intense study and vigorous debate in glycogen focused on the mechanisms controlling the initiation of glycogen biosynthesis. Since the discovery of phosphorylase by the Coris in 1939, it became clear that a primer was required for the synthesis of glycogen from individual glucose residues. The exact nature of this primer remained elusive for decades, despite intense scientific scrutiny. The determined effort of William Whelan over the course of decades finally bore fruit when he discovered that a protein, which he christened “glycogenin” was the primer of glycogen synthesis. The first part of this review will provide a detailed account of how this discovery was made and the insights into the process of glycogen synthesis that it revealed.

Close on the heels of the discovery of glycogenin followed the development of a new line of investigation into the structure and size of the glycogen particle within the cell. This area of investigation led to the proposal that glycogen was not a unitary molecule but rather was composed of distinct pools that could be separated based on solubility in acid and molecular

weight. A nomenclature came to be developed in the literature for these glycogen particles of differing molecular weight: the smaller particle was named “proglycogen” and the larger one “macroglycogen.” The idea that there could be different forms of glycogen that are actually distinct metabolic entities has been controversial. The debate over whether or not there are different species of glycogen remains unsettled. The second part of the review examines the development of the proglycogen/macroglycogen hypothesis and reviews the literature supporting and challenging this concept.

Following the discovery of glycogen phosphorylase, scientists believed for several years that this enzyme controlled both the synthesis and the degradation of glycogen. It was not until 1957, almost twenty years after the discovery of phosphorylase, that Luis Leloir published his classic study that showed that a newly discovered enzyme, glycogen synthase, catalyzed the buildup of glycogen via the formation of glycosidic bonds between glucose residues using UDP-glucose as the activated glucose donor. This was a pioneering moment in biochemistry not only because it revealed insights into the process of glycogen biosynthesis but because it was the key discovery that led to an understanding of the role of nucleotide sugars in biochemistry. Glycogen synthase has been studied extensively since its discovery and the examination of this enzyme over the course of several decades has led to greater understanding of mechanisms of metabolic control, including reversible phosphorylation and allosteric control. Study of glycogen synthase also contributed to our understanding of the mechanisms by which hormones such as insulin, glucagon, and epinephrine exert control over metabolism.

Branching enzyme is required for the synthesis of the glycogen macromolecule. Branching is essential to the structure of glycogen; it is the degree of branching seen in the structure of glycogen that differentiates it from the other major form of storage carbohydrate

encountered in nature, starch. Without the action of the branching enzyme, the glucose polymer formed by glycogen synthase demonstrates poor solubility within the cell and precipitates, acting as a foreign body. Deficiency of branching enzyme results in glycogen storage disease type IV, which is invariably fatal, usually by age 2 in humans. Despite the lethal consequences of a deficiency of branching enzyme, the enzyme remains poorly understood in terms of its regulation. Branching enzyme has only been the subject of one study pertaining to exercise, which reported an increase in the activity of the enzyme in response to an exercise training program (*3*). Any implications of this study have been largely ignored and the findings of the study have not been replicated or extended. The review will devote a section to a detailed examination of glycogen branching enzyme and will assert that glycogen branching enzyme should be studied in more detail. It is of interest to discover more about how glycogen branching enzyme is regulated. The current state of knowledge about the enzyme has developed from clinical medicine and biochemical studies that have revealed details about the structure of the enzyme and its catalytic properties, as well as the development of improved assays and clinical screening procedures to detect disease. However, the enzyme is underappreciated in terms of how it might be regulated as an adaptation to exercise. This review will set out to place the enzyme in context alongside other glycogen cycle enzymes and establish that the enzyme merits further study.

The review will then turn its attention to the degradative arm of glycogen metabolism. Glycogen phosphorylase, the rate-limiting enzyme in glycogen breakdown, was the first allosteric protein ever discovered. Like glycogen synthase, its study has led to the discovery and greater understanding of biological control mechanisms of general significance, such as reversible phosphorylation, allosteric control, and hormonal control.

Finally, the review will examine a critical enzyme in the degradation of glycogen, glycogen debranching enzyme. Glycogen phosphorylase cannot carry out the complete breakdown of glycogen for metabolic energy. The debranching enzyme is required in order for the phosphorylase to continue its catalytic action when it approaches four glucose residues away from a branch point. As one of the only enzymes to show dual catalytic activities (it is a transferase and a glucosidase), debranching enzyme has been of interest to biochemists. A deficiency of debranching enzyme causes glycogen storage disease type III, which causes liver and muscle pathology but has a milder clinical course than that shown in glycogen storage disease type IV, which is a deficiency of the branching enzyme. Like the branching enzyme, debranching enzyme has been studied mainly in clinical medicine and biochemistry. Little is known about the regulation of the enzyme, as it is thought not to be rate limiting in glycogen degradation. However, one study showed that the enzyme increases its activity in response to an exercise training program (*4*). This does provide evidence that the enzyme is regulated and thus could be important as an adaptation to exercise. Like branching enzyme, debranching enzyme could provide a worthwhile subject for further study.

The final part of the review will consider in more detail the rationale for a study of glycogen branching enzyme. Some possible candidates for experimental models will be explored. An assay has been developed for branching enzyme activity that was not available at the time that the only study relating branching enzyme to exercise was performed. The availability of this improved assay is another reason to revisit branching enzyme in both an exercise and dietary context.

Glycogen Biosynthesis

Glycogenin

After the discovery of glycogen phosphorylase in 1939 by Cori and Cori (*5*), the idea that this enzyme catalyzed both the synthesis and the degradation of glycogen *in vivo* persisted until the discovery of glycogen synthase by Leloir and Cardini in 1957 (*2*). Prior to the discovery of glycogen synthase, research revealed that glycogen synthesis did not proceed to a significant extent *in vitro* in the presence of glycogen phosphorylase and glucose 1-phosphate alone without the addition of glycogen. This observation prompted the hypothesis that a primer must be necessary for the initiation of glycogen synthesis. Following the discovery of glycogen synthase, intensive efforts began to determine the smallest saccharide capable of supporting *de novo* biosynthesis of glycogen by this enzyme.

Although research performed throughout the 1960's revealed much about the structure, function and regulation of glycogen, the molecular means by which the glycogen molecule initiated its growth remained obscure. Early indications were that simple maltosaccharides could, at least in vitro, act as the primer for glycogen synthesis and that maltotetraose was the smallest such saccharide that could prime efficiently (*6*). However, further investigation into a biosynthetic pathway for a maltosaccharide that could act as a primer for glycogen synthesis in vivo did not lead to the discovery of a credible mechanism for the formation of such saccharides. Some laboratories reported that glycogen could be synthesized without a primer. The data from these experiments led to the proposal that the de novo formation of glycogen could proceed when glycogen phosphorylase and glycogen synthase were mixed with glucose 1-phosphate and UDP-glucose respectively. These experiments could not, however, exclude the possibility of contamination of the system with primer (*7*).

Despite the first mention in 1886 by Richard Külz (*8*) of the possibility that glycogen was linked to a protein, substantial progress toward establishing this linkage was not made until the 1970's when Krisman and Barengo (*9*) authored a breakthrough study demonstrating that the primer in glycogen synthesis is a protein, which they named "glycogen initiator synthase." Their experiments revealed that when radioactive UDP-glucose was incubated with a liver extract a glycogen-like product was formed that precipitated when mixed with trichloroacetic acid (TCA) and contained radioactive glucose. The fact that the polysaccharide product formed in this reaction precipitated when mixed with acid demonstrated that a protein was covalently linked to the primer molecule necessary for the initiation of glycogen synthesis.

Subsequent experiments then extended the results of the previous work of Krisman and colleagues to reveal that in vitro synthesis of glycogen in enzyme systems from rat heart (*10*) and from *Escherichia coli* (*11*), *Neurospora crassa* (*12*), and bovine retina (*13*) also yielded glycogen bound to a protein. The work done over this period established that the glycogen primer was a protein, but the nature of the linkage between the carbohydrate and protein moieties of the glycogen macromolecule remained elusive. In 1988, a series of undertaken by Whelan and colleagues culminated in the discovery of the nature of the protein-carbohydrate linkage (*14*). After years of effort, the results of these experiments revealed a novel glucose to protein bond with the linkage formed between the glucose and the phenolic group of a tyrosine residue on the protein (*15*).

As multiple laboratories continued work on discovering more about the structure and function of this protein primer, more about its nature came to light. Whelan and colleagues christened the primer "glycogenin" before the protein had been purified and its structure elucidated. Soon after Whelan's laboratory had discovered the novel tyrosine-glucose linkage

that bound glycogenin to glucose, Cohen and Campbell then were able to sequence glycogenin from rabbit skeletal muscle and the primary structure from one species had thus been determined (*16*).

Since the 1980's, during which time decades of investigation culminated in the finding that glycogenin was the primer of glycogen synthesis, the pace of progress in discovering the mechanism by which glycogenin operates and its regulation has accelerated. The laboratories of Whelan and Cohen (*14, 17*) independently isolated muscle glycogenin and found that the protein could both glycosylate itself and prime glycogen synthesis – glycogenin and Krisman's "glycogen initiator synthase" were discovered after almost two decades of work to be one and the same (*18*).

Since the elucidation of the structure of glycogenin, research efforts have yielded new insights into how this protein initiates the biosynthesis of glycogen and we have begun to understand the role played by glycogenin in determining the rate of glycogen synthesis and the structure of the glycogen that is formed. Glycogenin that is unbound to carbohydrate (apo-glycogenin) has the catalytic ability to self-glycosylate, with Mn^{2+} as an activator. Once the growing glycogen molecule reaches a size of about 10 glucose residues (*19*), bulk glycogen synthesis can then proceed through the actions of glycogen synthase and glycogen branching enzyme. There is variation in the number of glucose units that the glycogenin attaches to itself by self-glucosylation, with the number of glucose residues added ranging from 7-11 (*20*). Evidence indicates that glycogen functions as a dimer in solution, with one subunit adding glucose residues to its partner (*21*).

Lines of inquiry that developed around the same time as the discovery of the catalytic mechanism of glycogenin led to the idea that different forms of glycogenin were specific to different species and tissues. Initial evidence for the existence of a second mammalian glycogenin-like species came from cDNA cloning (*22*). Definitive evidence for a distinct isoform of glycogen appeared in 1998 when Roach and colleagues characterized glycogenin-2, which they detected in human liver extracts (*23*). Further work indicated that this novel isoform of glycogenin was found primarily in liver tissue, but also to a lesser extent in the heart and pancreas, but not muscle (*22*). Interestingly, there is evidence that glycogenin-2 is confined to primates (*24*). This goes a long way toward explaining why the existence of this novel species of glycogenin remained obscure for as long as it did since research since the discovery of glycogenin had not focused on primates but rather on rabbit skeletal muscle and other tissues in non-primates.

The study of glycogenin opened interesting questions about how glycogen accumulation within the cell is regulated. Among other things, as more information became available about glycogenin, evidence emerged suggesting that glycogen existed *in vivo* in distinct pools that were separable by molecular weight and acid-solubility. This observation led to the naming of a low molecular weight glycogen particle that appeared to be a stable intermediate in glycogen synthesis: "proglycogen" (*25*). Subsequent sections of this review will provide more detail about proglycogen.

Naturally, the discovery of glycogenin raised questions about its possible role as a regulator in the rate of glycogen synthesis. Despite the glycogenin's attractiveness as a candidate in controlling the rate of flux during glycogen synthesis, experimental evidence has not supported a role for glycogenin as a limiting factor in attaining maximal glycogen levels. Data

obtained from experiments performed with rats and humans did not reveal a correlation between glycogen levels and glycogen amount or activity in muscle nor was there an observable increase in either glycogenin-1 protein or mRNA expression at rest, at exhaustion, or during recovery from exercise (*26, 27*).

Other avenues of research interest into glycogenin include the discovery of a protein that seems to play a role in regulating glycogenin itself. This protein has been named glycogen interacting protein (GNIP). GNIP appears to play a role in stimulating glycogenin by changing the spatial orientation of the glycogenin dimers, which could be necessary for initiating the self-glucosylation reaction (*28, 29*). It is also of interest to know whether or not glycogenin can translocate within the cell. An ability to change location in response to changing conditions within the cell could indicate that glycogenin could be recruited to areas where glycogen could be in high demand. Data has emerged showing that GNIP and glycogenin can bind to desmin and actin, respectively, and this could provide a means by which these proteins are recruited to specific locations within the muscle according to glycogen demand (*29, 30*).

The discovery that glycogenin is the primer for glycogen synthesis was a landmark finding that led to a greatly enriched understanding of exactly how glycogen synthesis begins. Along with the elucidation of its structure and catalytic mechanism, learning more about glycogenin prompted fruitful inquiry into determining how the rate of glycogen synthesis is controlled and in what subcellular locations the glycogen granule is formed. Questions about the regulation of glycogenin activity remain open and further investigation into the possible ability of glycogenin to translocate within the cell offer some directions for further study that will add to general understanding of glycogen's role in metabolism.

Proglycogen and Macroglycogen

As work proceeded investigating the nature of the primer in glycogen biosynthesis, interesting observations about the structure of the glycogen macro-molecule began to surface. Glycogen appeared to exist not as a singular molecular entity, but in distinct pools. Early reports indicated that glycogen turnover in skeletal muscle and liver did not proceed at a constant rate and that the differing rates of glycogen breakdown observed bore a relationship to the differing molecular weights of the glycogen granules within a given tissue (*31, 32*).

Long experience had shown that different chemical techniques for isolating glycogen from tissue extracts yielded differing amounts of glycogen. Isolating glycogen from tissue samples with hot alkali was a time-honored practice (notwithstanding how such treatment degrades proteins and set the search for glycogenin back by decades, perhaps). It had also been clear dating back to at least the 1950's (*33*) that glycogen particles of different molecular weights varied in their solubility in acid (*34*). Authors also noted that the amounts of acid-soluble glycogen differed according to the tissues from which glycogen was isolated (*6, 16, 25, 31-34*). In the early stages of research on glycogen, the observed variations in the amount of glycogen isolated from different tissues by using either hot alkali or dilute TCA led to the development of a bewildering terminology for glycogen. The fraction of glycogen isolated by hot alkali (KOH) treatment came to be known as "fixed," "bound," "desmo-," and "difficultly extractable." On the other hand, the fraction isolated by cold TCA precipitation was identified variously as "free," "lyo-," "trichloroacetic-acid extractable," and "easily extractable" (*35*).

It became clear during this time that the liver and skeletal muscle differed noticeably in the amount of "fixed" and "free" glycogen that could be extracted from each tissue. The distribution of glycogen in liver classified by molecular weight exhibited a preponderance of the

"free" glycogen, while in skeletal muscle the "fixed" glycogen was the more abundant form (*33*). Work progressed during the 1950's largely influenced by the classification scheme of "fixed" and "free" glycogen until experiments done in 1960 led to the virtual abandonment of the idea that there were structurally and functionally distinct pools of glycogen. Roe and colleagues used different homogenization techniques to examine the fractions of liver, muscle, and heart glycogen that could be extracted by TCA precipitation without hot alkali compared to that fraction which could be isolated using additional KOH following TCA precipitation. They found that if homogenization beads were used then the additional step of hot alkali treatment yielded no more glycogen than the amount extracted by cold TCA precipitation alone (*35*).

Investigation of differing pools of glycogen classified according to acid solubility reached an apparent zenith at the end of the 1950's. After the experiments of Roe and colleagues (*35*) questioned whether the extraction procedure might have been a source of error in experiments suggesting distinct species of glycogen, the concept of "desmo-" and "lyo-" glycogen fell from favor. Prevailing opinion shifted away from the notion that glycogen existed in metabolically distinct pools. Prior experimental evidence supporting this paradigm was dismissed as artifact arising from methods used to isolate glycogen from tissue samples. At this point, work on the topic of separate pools of glycogen classified by acid solubility largely ceased.

As work progressed on revealing the nature of glycogenin, however, some laboratories began to revisit the hypothesis that there were metabolically and structurally distinct species of glycogen. The discovery that the protein glycogenin was indeed the primer of glycogen synthesis not only validated the hypothesis proposed in 1886 by Külz that glycogen was bound to protein but also led to the postulation of a distinct type of glycogen that recalled Wilstätter's original

hypothesis of desmo- and lyo-glycogen (*36*). This time a new term would be introduced to the scientific lexicon: "proglycogen."

The work performed in the laboratory of Whelan and co-workers proved instrumental in formulating the concept of proglycogen and macroglycogen. In the course of their experiments leading to the discovery of glycogenin, it became clear that glycogenin existed as a covalently bound component of glycogen, linked to glycogen by a novel glucose linkage to tyrosine 194 on the glycogenin protein. However, Whelan also identified a glycogen-free form of glycogenin that, upon purification, proved to be an autocatalytic protein (*14, 17*). They referred to this glycogenin-like protein as self-glucosylating protein (SGP). Further work examining SGP revealed that filtered muscle extracts contained a protein that underwent glucosylation but that SDS-PAGE and radioautography demonstrated that the glucosylated protein was not SGP but rather a molecule with a much higher molecular weight of approximately 400 kDa. This entity, which they called p400, then became a target of investigation in an effort to clarify the biosynthetic pathway leading from glycogenin to "classic" or depot glycogen.

Preliminary observations from experiments done with p400 showed that when the molecule was incubated with UDP[^{14}C]glucose there was a resulting breakdown of p400 into a series of glucoproteins with molecular weights descending down to 37 kDa (p37), the same as glycogenin. P400 also proved to be subject to breakdown by amylase, which accelerated the conversion of p400 into p37 (*25*). Prior to the finding of p400 in muscle extracts, Whelan's laboratory had discovered its presence in adipocytes and a rat mammary tumor but there was no apparent connection to glycogenin because there was no breakdown of p400 into p37 in these tissue sources (*37*).

At this stage, the discovery of glycogenin and the identification of glycogen-like molecules of different molecular weights revealed by gel electrophoresis and separable by acid solubility lent credence to speculation that there might be discrete pools of glycogen that exhibit distinct metabolic behavior. The next set of experiments undertaken by Whelan and colleagues helped to clarify the path from p37 to p400 and concluded that p400 was a distinct species of glycogen.

Extracts of rabbit skeletal muscle were glucosylated in the presence of various activators and inhibitors of glycogen synthase and SGP. The data from these experiments led the authors to conclude that glucose residues were added to p400 by a glycogen synthase-like enzyme and also that p37, a lower molecular weight glucoprotein, was glucosylated by a different enzyme acting by autocatalysis. This set of experiments also provided evidence that glycogenin does not exist in the free state in skeletal muscle but rather is present in p400 and macro-molecular glycogen concurrently. The overall picture that emerged from this data was that glycogenin is built up by adding glucose residues by autocatalysis up to molecular weight of approximately 50 – 80 kDa and then grows further by adding glucose residues in reactions catalyzed by a synthase. SDS-PAGE and TCA precipitation allowed the isolation of a seemingly distinct pool of glycogen of a low molecular weight (400 kDa) that is acid-insoluble. This pool of glycogen, referred to previously as p400, was designated “proglycogen” (*25*).

Several implications arose out of this hypothesis of distinct pools of glycogen. Whelan suggested that different forms of glycogen synthase might be acting to synthesize proglycogen from SGP and glycogen from proglycogen. The key observation supporting this conclusion was that the synthesis of proglycogen from SGP proceeded rapidly in the presence of glucose 6-phosphate and micro-molar concentrations of UDP-glucose whereas the glucosylation reactions

required for the synthesis of the classic macro-molecular glycogen by glycogen synthase requires milli-molar concentrations of UDP-glucose (*25*). Additional support for the concept of a different enzyme building up proglycogen from SGP was provided by demonstrating that glucosylation of SGP proceeded rapidly in the presence of micro-molar UDP-glucose but required a different activator than the synthase, Mn^{2+} (*14, 17*).

While the structure of proglycogen seemed similar to glycogen, results from the 1990 experiments suggested that proglycogen is glucosylated preferentially in the presence of glycogen (*25*). The possibility also emerged that any assay of glycogen in tissue represents an average of pro- and macroglycogen and that new assays for each type of glycogen should be devised. The report of Whelan and colleagues in which the authors coined the term "proglycogen" and put forth the idea that a "proglycogen synthase" could be acting selectively to assemble proglycogen from SGP was a milestone in the study of glycogen biosynthesis. This new perspective fostered the development of new lines of inquiry that significantly refined our understanding of how glycogen synthesis proceeds; it also opened up a debate about the concept of pro- and macroglycogen that continues currently.

Several laboratories extended and refined the hypothesis proposed by Whelan that glycogen exists in distinct pools distinguishable by molecular weight, acid solubility, and metabolic behavior within the cell. One of the first studies that clarified the steps in the synthesis of glycogen from SGP to macroglycogen was carried out once again in the laboratory of Whelan and colleagues. Using cultured astrocytes from rats, the authors provided evidence that the path of glycogen synthesis followed this sequence: glycogenin → proglycogen → macroglycogen (*38*). These experiments, which used NH_4^+ in combination with re-feeding of [^{14}C] glucose to glycogen-depleted astrocytes, supported the proposition that proglycogen is a stable intermediate

in glycogen synthesis and that glycogen-depleted astrocytes begin the rapid re-synthesis of glycogen by glucosylation of SGP to form proglycogen. In the presence of NH_4^+, glycogen synthesis proceeded up to the point of proglycogen, but no further. In astrocytes untreated with NH_4^+, synthesis proceeded from proglycogen to macroglycogen. This observation led the authors to conclude that different glucosyl transferases act to catalyze each pathway. The experiments also provided evidence of a clear precursor-product relationship between proglycogen and macroglycogen.

After the publication of Whelan's experiments using astrocytes as a model system, other laboratories began to examine several aspects of glycogen metabolism from perspectives that reflected a growing acceptance of the concept of pro- and macroglycogen. One of the most prominent lines of inquiry concerned the process of glycogen biosynthesis under different conditions in skeletal muscle. These studies refined understanding of the time course of glycogen re-synthesis after glycogen depletion and also helped to clarify steps in the process of glycogen synthesis *in vivo* from glycogenin to macro-molecular glycogen.

While the question of whether proglycogen and macroglycogen are separate molecular entities remained open, studies using the device of separating glycogen into different fractions according to molecular weight and acid solubility proved useful in revising accepted concepts of glycogen synthesis. Prior to Whelan's proposal of the proglycogen concept, prevailing knowledge maintained that glycogen synthesis was a unitary phenomenon regulated mainly by glycogen synthase. The rate of glucose uptake into the cell had also been recognized as a regulator of the rate of glycogen synthesis (*39-43*).

The concept of proglycogen – macroglycogen led to development of a more sophisticated picture of glycogen synthesis. The work of laboratories such as that of Graham and colleagues revealed in greater detail the biphasic (*44*) character of glycogen synthesis in skeletal muscle (*45-49*). These experiments, along with the work of Huang and colleagues (*50*), provided evidence that in glycogen-depleted muscle the early phase of glycogen repletion results from an increase in the quantity of proglycogen. As glucose uptake continues and glycogen synthesis proceeds, the ratio of macroglycogen to proglycogen increases. Adamo and Graham (*49*) reported that the MG:PG ratio changed from 13:87 to 25:75 as the total glycogen concentration increased from 43.8 mmol/kg dw to 340.2 mmol/kg dw. This finding was in accordance with earlier observations by Jansson (*34*) comparing acid-soluble and insoluble fractions of glycogen in human skeletal muscle before the term proglycogen had been introduced into the literature. As glycogen concentration increases, there seems to be a glycogen concentration (350 mmol/kg dw) above which increases in total glycogen appear to be mainly due to increases in macroglycogen. At low glycogen concentrations, several studies have provided evidence that proglycogen is more dynamic than macroglycogen (*46, 48-52*). This body of evidence indicates that in glycogen-depleted muscle, the restoration of glycogen stores follows a time course during which proglycogen is replenished before macroglycogen and that as glycogen stores are built up, macroglycogen represents an ever greater portion of the total glycogen concentration. The buildup of glycogen stores to levels greater than normal resting levels, known as supercompensation, appears to result from the accretion of macroglycogen rather than proglycogen (*52*).

The introduction of the proglycogen - macroglycogen hypothesis also stimulated interest in how the different fractions of glycogen respond to exercise. Three hypotheses were candidates

to explain how glycogen fractions could be utilized during exercise. The first hypothesis held that proglycogen is the most dynamic fraction of glycogen and is utilized preferably as a substrate for exercise while macroglycogen exists as a relatively inactive storage form. The second hypothesis maintained that proglycogen is a stable intermediate and that glycogenolysis breaks down macroglycogen as a preferred fuel source with proglycogen as a stopping point. Alternatively, proglycogen and macroglycogen are used at equal speeds and the breakdown of each is not a consequence of any functional difference between either fraction but is determined rather by the availability of each.

The body of work done to this point regarding regulation of glycogen metabolism during exercise leaves several questions open, despite the progress made over the last decade. The relative contributions of the proglycogen and macroglycogen fractions as a substrate for exercise appear to be contingent upon initial glycogen concentration before the onset of exercise, the intensity level of the exercise, whether the exercise is single- or repeated-bout, and the species of animal under investigation.

The general picture emerging is that when performing mild to moderate intensity endurance exercise, proglycogen and macroglycogen can both be utilized, with initial glycogen concentration being a significant factor determining the extent to which each glycogen fraction is degraded (*52-59*). Derave and colleagues found that in rats initial macroglycogen concentration, but not that of proglycogen, was correlated with the rate of glycogenolysis in electrically stimulated skeletal muscle (*53*).

The species under study is a source of variation in degradation of the different glycogen fractions during exercise, with horses seeming to rely to a greater degree on macroglycogen than

humans (*56, 57*). Data also suggest that horses store a greater amount of macroglycogen than humans (*56*). Data from humans indicates that proglycogen is the more dynamic of the two fractions in that the rate of glycogenolysis during exercise appears to be greater in the proglycogen fraction at all levels of exercise intensity studied thus far (*54, 55*). Endurance exercise of a long duration (marathon running) has to date been the only exercise modality studied that has resulted in macroglycogen being degraded to a greater degree than proglycogen in humans (*52*). Shearer and colleagues have also reported that macroglycogenolysis in humans can be greatly suppressed when repeated bouts of exercise are performed (*55*).

Various authors (*47, 54-57*) have also speculated that patterns of pro- and macrogenolysis are subject to a mechanism specific to muscle fiber-type. To date, the research done has been insufficient to clarify this question. Exercise protocols utilized have certainly resulted in the recruitment of Type I and Type IIa muscle fibers, with a study done by Brojer and colleagues on horses performing maximal treadmill exercise showing evidence that Type IIb fibers were also recruited (*56*). The authors speculate that proglycogen might be preferentially degraded in Type IIb fibers, which would explain their finding that greater breakdown of proglycogen occurred during maximal exercise in horses versus greater macroglycogen utilization during endurance exercise. In addition to fiber type, the heterogeneity in the location of the glycogen granule has been put forward as a possible factor determining glycogen degradation patterns in skeletal muscle (*60*).

Currently, there is evidence to support the conclusion that proglycogen is a dynamic source of substrate in exercising muscle under almost all conditions (*54, 55*), with macroglycogen being degraded under conditions of relatively moderate exercise intensity and during long duration endurance exercise in humans, when the metabolic demand of the activity

forces the mobilization of macroglycogen stores (*52*). High intensity exercise appears to increase the rate of breakdown of proglycogen (*54, 56*), although the underlying mechanism of this observation remains to be explained. The influence of muscle fiber type on the pattern of glycogen degradation is a topic that merits further investigation. Future studies in this direction might help to clarify the functional differences between the two fractions of glycogen and would help to settle the question of whether pro- and macroglycogen are under different enzymatic regulation, as proposed by Whelan and colleagues (*6, 25, 38, 61*).

The debate over whether proglycogen and macroglycogen are distinct molecular entities deserves mention before concluding the discussion on these two fractions of the glycogen pool. Several authors have challenged the hypothesis that proglycogen and macroglycogen represent different species of glycogen (*62-64*).

The main support for the concept of proglycogen lies in its insolubility in dilute acid, which is the basis for making a distinction between it and "classic" or depot glycogen, which is acid-soluble. The idea that proglycogen is of a discrete molecular weight of 400 kDa is also central to the proglycogen – macroglycogen paradigm. Experimental evidence challenging this hypothesis arises from an assertion that the glycogen extraction technique used by the laboratories performing the first several experiments with pro- and macroglycogen overestimated the amount of acid-insoluble glycogen and thus underestimated the fraction of "classic" glycogen (*63, 64*). The issue revolved around whether or not the muscle tissue extracts had been homogenized prior to the measurement of pro- and macroglycogen. Homogenization of the tissue extracts disrupts the tissue and would conceivably allow more of the total glycogen in the extract to be measurable. The authors using the homogenization treatment maintain that use of a homogenization-free extraction method fails to free acid-soluble glycogen from the sarcoplasmic

reticulum and the dense mesh of myofibrils where most glycogen particles are found (*65, 66*). James and colleagues also provide evidence that the acid-insoluble fraction of glycogen does not correspond to a pool of glycogen of low molecular weight (*66*).

Proglycogen, Macroglycogen, and Exercise

The preponderance of research on pro- and macroglycogen has not focused on exercise *per se*, however, a small body of literature has developed since the late 1990's that has sought to explain the roles of different glycogen fractions in exercise. The results of several studies on exercise and proglycogen and macroglycogen are summarized in Table 1.1 below.

Table 1.1 Summary of Studies with Exercise and Proglycogen/Macroglycogen

Author and year	Purpose	Subject Description	Results
Adamo et al, 1998	Determine whether two structural forms of glycogen (PG and MG) function as different metabolic pools	Nine human males	PG and MG metabolized differently in timing and magnitude, both are sensitive to diet
Asp et al, 1999	Assess how MG and PG fractions are restored after a marathon and to determine whether glycogen accumulates differently in various fiber types	Six marathon runners	MG is used to greater degree during marathon than PG and is slower to replete than PG after marathon; glycogen repletion delayed in slow-twitch fibers compared to fast twitch
Derave et al, 2000	Investigate relative contributions of PG/MG to glycogenolysis during muscle contraction	Rats (n=25)	Both PG and MG are degraded; when glycogen stores are amply available MG is preferentially utilized
Shearer et al, 2001	Examine relationship between pre-exercise muscle glycogen content and utilization of PG and MG	Six male humans	Initial glycogen concentration regulates glycogenolysis in PG and MG pools; PG preferentially utilized at onset of exercise when glycogen concentration is high. Lower glycogen levels and repeated exercise result in equal utilization of PG/MG

(Table cont'd)

Author and year	Purpose	Subject Description	Results
Graham et al, 2001	Assess changes in PG and MG under varying exercise intensities and durations	21 human males	PG appears more dynamic and is utilized preferentially in more situations than MG; rates of glycogenolysis consistently higher in PG fraction
Essen-Gustavsson et al, 2002	Study effect of an endurance race on PG and MG fractions	Seven endurance trained horses	MG was utilized to a greater degree than PG during an endurance race
Brojer et al, 2002	Investigate degradation of PG and MG during intense treadmill exercise	Ten standard-bred trotter horses	PG and MG contribute equally to glycogenolysis during intense exercise; resynthesis starts in PG pool
Brojer et al, 2006	Determine resynthesis of PG and MG in muscle after intermittent exercise	Nine trained standard-bred trotter horses	MG resynthesized faster during first 24 hrs post-exercise; equal rates of resynthesis in following 48 hrs

While new evidence has emerged to challenge the hypothesis that proglycogen and macroglycogen are distinct molecular entities, there is support for considering the molecular weight of glycogen fractions under certain conditions. While "proglycogen" and "macroglycogen" might best be viewed as purely operational terms and not separate species of glycogen, the introduction of the terms into the nomenclature has stimulated renewed interest in the mechanisms controlling the synthesis and degradation of glycogen.

Whether or not the fractions of glycogen referred to as pro- and macroglycogen are indeed separate molecular entities or even truly correspond to pools of glycogen that differ in molecular weight, data supports the idea that different fractions of glycogen respond differently to metabolic stimuli (*67*). The proglycogen concept has undoubtedly led to insights that reveal a significantly more complex regulation of glycogen than had been supposed even two decades ago. The data from the studies employing the proglycogen – macroglycogen paradigm have

pointed to new directions for understanding how glycogen is regulated. Recently, attention has turned toward investigating how the variable subcellular location of glycogen and its interaction with scaffolding proteins and enzymes control the flux of glycogen in response to changing metabolic conditions.

Glycogen Synthase

After the discovery of glycogen phosphorylase by the Coris in 1939, the prevailing knowledge at the time maintained that phosphorylase not only controlled glycogen degradation but also that this enzyme was responsible for glycogen synthesis. As work progressed, it became clear that *in vitro* synthesis of glycogen could not proceed to a significant extent when only glycogen phosphorylase and glucose 1-phosphate were present in the reaction mixture (*68*). This observation led to the conclusion that a primer was necessary for the synthesis of glycogen. While the elucidation of the primer for glycogen biosynthesis awaited the discovery of glycogenin by the team of W.J. Whelan in the 1980's, Leloir and Cardini discovered glycogen synthase in 1957 and thereby clarified that different enzymatic systems were responsible for the biosynthetic and degradative arms of glycogen metabolism (*2*). Since the discovery of glycogen synthase, the enzyme has been the subject of a voluminous body of scientific inquiry. This review will, however, primarily focus on the research pertaining to the role of glycogen synthase in exercise while also aiming to provide an overview of the work done pertaining to how GS is regulated.

Glycogen synthase is a key enzyme in glycogen synthesis. This enzyme catalyzes the addition of glucose residues in a linear chain bound by alpha 1-4 linkages between individual glucose units, with UDP-glucose as the activated glucose donor molecule. Regulation of GS is

complex and is mediated by several kinases and phosphatases that covalently modify the enzyme by reversible phosphorylation. Additionally, the enzyme is subject to allosteric regulation by the binding of glucose 6-phosphate (*69*).

The various signaling events that trigger modification of glycogen synthase by phosphorylation are coordinately regulated by variable hormonal concentrations. Insulin, glucagon, catecholamines, and glucocorticoids are all involved in regulating the activity of glycogen synthase and allow for flexible and finely tuned regulation of glycogen synthesis under highly variable conditions. The energy charge of the cell as determined by the AMP/ATP ratio and the nutritional status of the organism are two key stimuli that regulate the activity of GS. The concentration of glycogen is also a potent control mechanism of GS activity. High glycogen concentrations inhibit GS, while low glycogen concentrations stimulate GS activity. There is also evidence that the location of GS within the cell is not fixed and that GS translocates to different subcellular locations dependent on the relative abundance or scarcity of glycogen depots within the cell and the cell's energy state (*70*). The following section will highlight some of the more important work that has been done in elucidating the many levels of regulation of GS.

Glycogen Synthase Regulation

Glycogen synthase has been viewed as the rate-limiting step in the synthesis of glycogen, although there is still debate regarding whether glucose transport or glycogen synthase is truly the flux controlling step (*19, 71*). Although this question has not yet been conclusively settled, a large body of evidence indicates that GS contributes significantly to controlling the rate of glycogen synthesis. Therefore it is important to provide an overview of how this enzyme is

regulated in an effort to place GS in its proper context within a broader discussion of glycogen metabolism.

Glycogen synthase regulation is complex and the mechanisms controlling the enzyme are not fully understood. The complexity of GS regulation reflects the important role of GS in metabolic regulation. GS activity is known to be regulated by reversible phosphorylation and allosteric activation by glucose 6-phosphate. Several external stimuli such as insulin, catecholamines, glycogen content, and exercise also exert an influence on GS activity by changing phosphorylation state and the concentration of allosteric activators.

One level of control of GS is reversible phosphorylation. Phosphorylation of GS inhibits the enzyme. Investigators have thus far identified nine well-documented phosphorylation sites (*72-74*). While an exhaustive list of all the kinases that phosphorylate GS goes beyond the scope this review, some of the kinases reported to phosphorylate various sites in GS include: GSK-3, AMPK, PKA, phosphorylase kinase, CaM-kinase II, and casein kinase (*72, 73, 75-77*). Among these kinases, GSK-3, AMPK, and PKA all have physiological significance in regulating GS. It is noteworthy to mention that the nine phosphorylation sites identified on GS theoretically allow for 512 possible combinations of phosphorylation configurations, but due to hierarchal phosphorylation it is highly likely that there are far fewer combinations possible *in vivo* (*72*). Even so, the number of possible phosphorylation combinations still allows for complex layers of regulation of GS and possible redundancy of control of the enzyme. Such intricate regulation of GS is evidence of the central role played by the enzyme in glycogen metabolism.

Although a complete understanding of the roles played by these several kinases in phosphorylating GS has not yet been attained, research efforts have succeeded in characterizing

how some of the phosphorylation sites influence activity. In particular, the laboratories of Cohen and Roach have provided insight into the actions of epinephrine and insulin in modifying the phosphorylation of several serine residues on GS. Epinephrine increases phosphorylation of several serine residues, thus reducing GS activity, while insulin reduces phosphorylation of several of the same serine residues, thereby increasing activity of the enzyme (*72, 73*).

While a significant amount of research attention has focused on the phosphorylation state of GS and the many kinases that phosphorylate GS, it is also important to recognize the role played by glucose 6-phosphate in regulating the activity of GS. GS activity in the presence of high levels of glucose 6-phosphate remains high regardless of the phosphorylation state of GS. However, the concentration of glucose 6-phosphate required to stimulate GS activity to its maximal rate is far above the physiological range of 0.1-0.2mM in muscles (*74*). The sensitivity of GS to glucose 6-phosphate is also controlled by the form of GS, which predominates in a tissue at any given time. GS is known to exist in two interconvertible forms. The I-form of the enzyme is active independently of glucose 6-phosphate. The inactive D-form, on the other hand, is dependent on glucose 6-phosphate for its activation. The conversion of GS between the two forms is dependent on the phosphorylation state of the enzyme, which is determined by hormonal signals from insulin and epinephrine, glycogen content, and muscle contraction. Insulin activates GS and increases glycogen synthesis by decreasing phosphorylation of GS and by increasing the concentration of glucose 6-phosphate. Epinephrine inhibits GS and reduces glycogen synthesis by increasing phosphorylation of GS and blocking insulin-mediated activation of GS. Epinephrine also reduces the affinity of GS for its allosteric activator, glucose 6-phosphate. Glycogen content and muscle contraction also play significant roles in regulating GS.

The study of the interplay of these factors in regulating GS has led to the development of insights into glycogen metabolism and has prompted inquiry into which of the various control mechanisms are most important in controlling the net rate of glycogen synthesis. A particularly active area of research has focused on the relative importance of glucose transport into the cell versus the action of glycogen synthase as the dominant flux-controlling step in the synthesis of glycogen.

While GS has been viewed as the rate-limiting step in glycogen synthesis, challenges have emerged to this concept, proposing that glucose transport and phosphorylation of glucose by hexokinase are the rate-limiting steps in glycogen synthesis. The experimental basis for this assertion has arisen largely from the work done in the laboratory of Shulman and Rothman. Using nuclear magnetic resonance (NMR) to conduct in vivo experiments using humans and rodents, the authors concluded that glucose transport and not GS was the flux-determining step (*78-80*). They based their conclusions on the experimental findings from their work and several other studies that indicated a mismatch between the rate glycogen synthesis *in vivo* and the rate attributable to the degree of phosphorylation of GS observed *in vitro* (*81-83*). From these data, the authors propose that by taking into account the degree of phosphorylation of GS in conjunction with the *in vivo* concentrations of glucose 6-phosphate and ATP a quantitative agreement between the *in vitro* rate of glycogen synthesis observed in the GS assay and the in vivo rate observed during NMR studies can be obtained. The conclusion that glucose 6-phosphate concentrations are critical in determining the rate of glycogen synthesis *in vivo* is to say that glucose transport is a rate-limiting step in glycogen synthesis. This is due to the fact that glucose 6-phosphate concentration in the cell is limited by glucose transport because glucose is the substrate for hexokinase, which phosphorylates glucose immediately upon entry into the

muscle cell, thereby trapping the glucose within the cell. From this point, glucose 6-phosphate can undergo many fates within the cell and is the key allosteric activator of GS.

Experimental evidence also supports a role for GS as the flux-determining step in glycogen synthesis. Experiments with transgenic mice overexpressing an active form of glycogen synthase showed that the animals expressing the mutant GS had a marked increase in glycogen synthesis independently of glucose transport compared to controls (*84*). Further evidence supporting a rate-determining role for GS came from the in vivo glucose-clamp analyses of Rossetti and Hu (*85*). These experiments showed that GS activation occurs *in vivo* at insulin levels lower than that required to stimulate glucose uptake and that even at insulin concentrations that were insufficient to result in glucose uptake or glycogen accumulation GS activity was sufficient to prevent net glycogenolysis by stimulating reincorporation of glucose equivalents into glycogen. These results indicate that GS activity is important in controlling flux of glucose into glycogen independent of glucose transport. It has also been noted that increased insulin decreases both UDP-glucose and glucose 6-phosphate prior to activation of glucose transport (*85*). This observation supports the notion that insulin-activated GS can "pull" glucose in the direction of glycogen synthesis before glucose is "pushed" into the cell by insulin-activated glucose transport. This concept is supported by experiments with cut muscle fibers, which allow glucose entry by simple diffusion (*86*). When glucose uptakes were held constant by varying the concentration of glucose in the medium, glycogen synthesis was higher in insulin-treated preparations, indicating that insulin-stimulated GS activity directs intracellular glucose into glycogen.

From the foregoing evidence, it is perhaps most prudent to conclude that both GS activity and glucose transport play a role in determining the rate of flux of glucose to glycogen. NMR

provides an attractive method for examining glycogen metabolism due to its being non-invasive and therefore well-suited to human experiments. Interpretation of the results from NMR studies can be difficult to interpret however, due to the limitation of the method in dealing with variations in the spatial distribution of metabolites in cells and tissues (*19*). There is substantial evidence for a central role of GS in controlling the rate of glycogen synthesis, although the interpretation of data from transgenic animals applied to control populations has caveats. It is difficult to control for compensatory mechanisms arising in transgenic animals as a response to genetic mutation that can confound the results of experiments and limits the generalizability of results to the broader population. Nonetheless, evidence does support the conclusion that GS does play a key role in controlling glycogen synthesis even when controlling for glucose uptake.

Glycogen Synthase and Exercise

Apart from the debate over the relative importance of glucose uptake versus GS activity in determining the rate of glycogen synthesis, another issue of importance in glycogen metabolism is the role of exercise in influencing glycogen flux, particularly in modulating the activity of GS and glucose transport. In the course of discussing this topic, the importance of glycogen content in skeletal muscle and the intracellular location of GS will also be mentioned since each relates to muscle contraction in regulating glycogen metabolism.

GS activity has been shown in several studies to be regulated by muscle contraction (*74, 87, 88*). Specifically, GS activity is increased after muscle contraction (*87*) and following exercise, glycogen synthesis is increased even in the absence of insulin (*89, 90*). The exact mechanisms underlying the increase in GS activity remain unclear. However, some authors report that contraction decreases phosphorylation of GS on several serine residues (*91, 92*). The

effect of muscle contraction on GS might also be mediated by protein phosphatase 1 (PP1), which activates GS by de-phosphorylating GS at specific serine residues (*93*).

While progress has been made in identifying possible mechanisms by which muscle contraction can stimulate GS and thereby increase glycogen synthesis, data has accumulated suggesting that the potency of exercise in modulating GS activity and glycogen synthesis might be the result of other factors not intrinsic to exercise *per se*. Evidence from several studies indicates that the increase in GS activation observed with exercise is due not to a change in the phosphorylation state of GS brought about by exercise acting independently but rather by a decrease in glycogen content in the muscle as a result of glycogenolysis during the exercise bout (*83, 87, 94*). Clearly, glycogen content itself is a potent regulator of its own synthesis and there is data to show that glycogen content can override other regulatory factors in determining the rate of synthesis (*94-98*).

A recent line of experimental inquiry has provided new insight into mechanisms regulating glycogen synthase. In their experiments with rats, Nielsen and colleagues reported that GS translocates from glycogen-rich membrane locations to the cytoskeleton in the skeletal muscle tissue of animals with low glycogen levels (*94*). The experiments also showed that high glycogen concentrations in skeletal muscle inhibited GS even in the presence of insulin, which is a potent activator of the enzyme. These experiments have pursued a theory, introduced as early as the 1960's, that glycogen was present in dynamic cellular organelles called "glycosomes" (*99*). This concept proposes that the linkage of glycogen with its regulatory proteins is critical to the function of glycogen in metabolism.

Experiments by Prats (*100*) and Nielsen (*94*) have investigated the translocation of GS within the cell and the data from this work has lent support for the glycosome concept and for the notion that the translocation of GS in response to the changing energy state of the cell is an important regulatory mechanism in controlling glycogen synthesis and cellular energetics. In particular, the work of Prats and colleagues (*100*) showed that after the metabolic stress of glycogen-depleting muscle contraction, GS undergoes redistribution within the cell from the nucleus to spherical structures associated with the actin cytoskeleton. This occurred within glycogen-depleted muscle fibers in a refractory period, indicating that these fibers were in the early stages of metabolic recovery and glycogen resynthesis. These findings also comport with prior data showing that GS requires binding to glycogenin for efficient initiation of glycogen synthesis, considering that glycogenin and GS have been found associated with the actin cytoskeleton (*94, 101, 102*).

The work done in the last two decades has provided much insight into the regulation of glycogen synthase by hormones, exercise, and glycogen content while leaving many questions yet unanswered. The mechanisms underlying the ability of muscle contraction to modulate GS remain unclear. Among the issues remaining unsettled is the exact nature of the relationship between contraction and PP1, the phosphatase that de-phosphorylates GS, thereby increasing GS activity. Indeed, data has accumulated calling into question the idea that muscle contraction independently activates GS, especially when glycogen content is high (*83, 94*). The role of GS movement within the cell in regulating glycogen metabolism is a topic only beginning to be studied systematically. Even the relative contribution of insulin to the overall regulation of GS is a subject of active investigation. The resolution of these questions will continue to present attractive targets of study in the future.

Glycogen Branching Enzyme

Glycogen branching enzyme (GBE) completes the synthesis of glycogen by catalyzing the formation of alpha 1-6 linkages and thereby creating branch points in the growing glycogen molecule. GBE transfers chain fragments from glycogen by the cleavage of an alpha 1-4 bond from a donor, previously catalyzed by GS, to an acceptor via the formation of an alpha 1-6 bond (*103*). The acceptor may or may not be the original donor. The reaction catalyzed by GBE does not result in any net glycogen synthesis, but is only a transfer reaction that modifies the structure of the mature glycogen particle.

One aspect of the physiological significance of the branched structure of glycogen is the enhanced solubility of glycogen conferred by branching. Without branching, the polysaccharide formed by GS precipitates and act as a foreign body within the tissues. Even though, according to the present state of knowledge about the degree of branching of glycogen, the alpha 1-6 linkages constitute only approximately 10% of the linkages in glycogen (*1*), a deficiency in branching enzyme results glycogen storage disease type IV, which is fatal in humans, usually by four years of age. Glycogen storage disease type IV will be discussed in more detail in a later section.

GBE has been studied extensively in clinical medicine and biochemistry due to its importance in determining the structure of glycogen and its significance as the cause of a severe, if rare, metabolic disease. The study of GBE in medicine and biochemistry has led to insights into the mechanism of action of the enzyme (*104-107*) and the development of improved assays for measuring the activity of the enzyme (*106, 108*). The bulk of the research effort into GBE of direct relevance in humans has focused mainly on the development of pre-natal screening for

glycogen storage disease type IV during pregnancy. Case reports on such aspects as heterogeneity in presentation of the symptoms of glycogen storage disease type IV have also appeared in the literature (*109-113*). However, the enzyme has received almost no attention in an exercise context.

Only one study in 1974 has examined GBE and its relationship to exercise (*3*). Taylor and colleagues reported that GBE activity is increased following a 12-week endurance cycling training program in a group of males ranging in age between 21-26 years. The participants were trained (n=8) and sedentary (n=8). Additionally, the authors noted that the activity of GBE decreased during prolonged sub-maximal exercise and then showed an increase in activity after the cessation of both prolonged sub-maximal exercise and maximal work to exhaustion. This pattern of activation corresponds to the activity of GS and is thought to be attributable to varying levels of insulin (*114, 115*). The findings of this study have not prompted any further investigation into the relationship between GBE and exercise. Of note in this particular study is the fact that the assay method for measuring branching enzyme activity was not described in any detail. A reference was made to a paper describing the method. However, the reference was listed as "in press" and was nowhere to be in found in the published literature after an intensive search.

The observation that GBE activity increases in response to an exercise-training program could be of more significance than has been appreciated thus far. Perhaps the general acceptance that GS activity and glucose transport are the rate-limiting steps in glycogen biosynthesis has been responsible for the fact that GBE has eluded scientific inquiry relating to exercise and nutrition. However, the findings of Taylor and colleagues clearly provide at least preliminary evidence that GBE activity is increased by exercise. While GBE is generally not regarded as

rate-limiting in glycogen synthesis, the increase in GBE activity demonstrates that there is an investment of biological resources in increasing the amount of branching activity in parallel with the increased activation of GS during the rapid phase of glycogen repletion following exercise. Also, the finding that GBE activity increases following an exercise training program of 12 weeks indicates that the transcriptional machinery is activated, leading to an increase in the concentration of GBE within skeletal muscle in response to training. It is logical to conclude that such increases are of functional physiological significance. The enhanced solubility of glycogen conferred by additional branching would allow for more glycogen to be stored within the muscle. Also, the formation of more alpha 1-6 linkages results in an increased number of terminal residues incorporated into the mature glycogen granule. Glycogen phosphorylase and glycogen synthase both act on terminal residues. In this manner, branching increases both the rate of glycogen synthesis and the rate of degradation. This is one important functional consequence involving exercise of the finding that GBE activity is increased in response to training (*3*).

Further data has accumulated in studies of genetically modified rodents indicating that GBE is of importance in regulating glycogen metabolism by ensuring that the structure of glycogen synthesized is properly branched. In mice modified to overexpress glycogen synthase, glycogen is not branched to the degree found in normal mice and polyglucosan bodies develop and precipitate within the tissues (*116, 117*). These findings occurred in animals that had no deficiency in GBE, at least in the sense that the mice did not have a mutation that caused a complete absence of GBE of the kind seen in type IV glycogen storage disease. On the other hand, the results indicate that even a mismatch between the relative activities of GS:GBE causes dysregulation of the degree of branching that results in malformed glycogen with a structure similar to that found in Andersen's disease (GSD-IV). Clearly, GBE must be regulated in such a

way that its activity remains proportional to that of GS. However, the control of the glycogen branching/debranching system remains poorly understood and is an area of glycogen metabolism that has not received the amount of attention that it probably merits.

Another aspect to consider in a discussion of the significance of GBE pertains to glycogen storage disease type IV, which is caused by a deficiency of GBE. The disease was first reported by Andersen in 1956 (*118*). The patient presented with massive enlargement and cirrhosis of the liver, with autopsy showing an accumulation of an abnormal glycogen with a structure appearing similar to that of amylopectin (starch). The clinical progression of the disease results in liver failure causing death, usually by age 2 (*119*). Work done after the initial reports of the disease confirmed that the cause was an inherited genetic defect (autosomal recessive) resulting in an absence of GBE (*120*).

Viewed in the light of the evidence accumulated from clinical medical research on glycogen storage disease type IV, the role of GBE in glycogen metabolism gains added perspective and significance. The severe clinical course of glycogen storage disease type IV shows that the action of the enzyme is necessary for life. The fact that GBE has not been viewed as rate-limiting in glycogen synthesis has perhaps diminished the attention paid to the enzyme while at the same time the severity of glycogen storage disease type IV has probably led to the emphasis of research on GBE to be focused on aspects of the enzyme related to clinical medicine. However, the one study performed by Taylor and colleagues did provide preliminary evidence that GBE increases in response to an exercise stimulus (*3*). Even if GBE is not rate-limiting to glycogen synthesis in the strictest sense of the term, an increase in GBE reveals that the enzyme is of relevance in adaptation to exercise.

Glycogen Degradation

Glycogen Phosphorylase

Glycogen phosphorylase (GP) catalyzes the regulated step in the breakdown of glycogen. GP catalyzes the phosphorolysis of alpha 1-4-glycosidic linkages of glycogen, yielding glucose 1-phosphate and shortened glycogen as its products (*19*). GP continues the phosphorolytic cleavage of alpha 1-4 linkages until it reaches a point 4 residues short of an alpha 1-6 linkage, the hydrolysis of which requires the action of a separate debranching enzyme. GP has been viewed classically as the rate-limiting enzyme in the breakdown of glycogen and the regulation of the enzyme has been the subject of intense investigation.

The discovery of glycogen phosphorylase by the Carl and Gerta Cori was an epoch-making event in history of biochemistry (*121*). GP was the first allosteric protein ever discovered and its study led to an understanding of mechanisms of general significance in biological science, such as allosteric activation and regulation of enzyme activity by reversible phosphorylation. Following the discovery of GP, for several years the enzyme was thought to be responsible for both the synthesis and degradation of glycogen (*1*). This view was revised when Leloir and Cardini first reported that glycogen synthase was the enzyme responsible for the synthesis of glycogen by adding glucose residues to the growing glycogen granule by the formation of alpha 1-4-glycosidic bonds with UDP-glucose as the activated glucose donor (*2*).

The study of GP led to new vistas in the understanding of metabolic control systems, particularly allosteric control and covalent modification. GP has been studied in detail such that the catalytic mechanism of the enzyme is known in molecular detail. The three-dimensional structure of the protein has been resolved from X-ray crystallographic studies and the changes in

the structure of the enzyme brought about by allosteric interactions and reversible phosphorylation are well understood.

GP exists in two forms in three different tissues in humans; liver, muscle, and brain (*122*). Phosphorylase *a* is the active form of the enzyme which does not require AMP for catalysis to proceed, although AMP does augment the rate of the enzyme by roughly 10% (*123*). Phosphorylase *b* predominates in resting muscle and is in the inactive T state, requiring binding by AMP for transition to the active R state (*124*). The transition of phosphorylase *b* from the T to the R state is also controlled by phosphorylation at a single serine residue (serine 14) by phosphorylase kinase. Conversely, phosphorylase *a* is deactivated by hydrolysis of the phosphorylated serine residue by protein phosphatase 1 (PP1). Glycogen phosphorylase and glycogen synthase are thus coordinately regulated by reversible phosphorylation, with GS being relatively inactive while phosphorylated at the same time that GP is relatively active while phosphorylated.

GP is also regulated by several other ligands and allosteric effectors. AMP, ATP, glucose 6-phosphate, epinephrine, glucagon, and insulin all exert an influence on the activity of GP by changing the phosphorylation state, and thereby the inter-conversion of the enzyme between the *a* and *b* forms. AMP, epinephrine, and glucagon all signal that the energy state of the organism is low and that glycogen should be mobilized. Conversely, ATP, glucose 6-phosphate, and insulin signal that fuel is abundant and thereby activate glycogen synthase while simultaneously shifting the relative concentrations of GP so that the inactive *b* form predominates.

Glycogen and glycogenolysis are important for exercise performance. Intense exercise can cause almost the complete depletion of glycogen stores (*125-127*). The essentiality of the

mobilization of glycogen as a fuel for exercise is underscored by the exercise intolerance experienced by individuals suffering from glycogen storage disease type V, which is caused by an absence of GP, first reported by Brian McArdle in 1951 (*128*). Patients suffering from McArdle's disease experience painful muscle cramps during exertion and are severely limited in their capacity for exercise. A 1972 study by Taylor and colleagues reported an increase in phosphorylase *a* following an endurance training program (*129*).

During exercise, GP activity increases. GP is thought to be activated in skeletal muscle by exercise mechanistically by the contraction-induced elevation of Ca^{2+} causing an activation of phosphorylase kinase, which phosphorylates GP and in turn generates the active phosphorylase *a* (*19*). Epinephrine enhances the activation of GP by initiating the cAMP signaling cascade. cAMP is a potent intracellular messenger and an activator of GP.

Glycogen Debranching Enzyme

Glycogen debranching enzyme (GDE), also known in the literature as amylo-1,6-glucosidase, 4-alpha-glucanotransferase (AGL), is a bi-functional enzyme acting in concert with glycogen phosphorylase to complete the degradation of glycogen. GP degrades the glycogen chains up until four glucosyl units before an alpha 1-6 branch point. GDE then carries out the transfer of three glucose residues from the short branch to the end of another branch using its transferase activity; the enzyme then hydrolyzes the alpha 1-6 branch point residue by using its amylo-1,6–glucosidase activity (*130*). For complete degradation of glycogen, the dual transferase and glucosidase activities of GDE and the activity of GP are required. Structural and biochemical studies have purified the protein and characterized GDE in molecular detail; these studies have

revealed the enzyme to be a large monomeric protein having a molecular mass of 165-175 kDa (*131-133*).

GDE has received almost no attention in exercise science. One study by Taylor and colleagues reported a two-fold increase in GDE activity following a 12 week endurance training program while observing that GDE activity decreased immediately following submaximal and maximal exercise to exhaustion (*4*). These observations were consistent with those of studies performed in the same laboratory with glycogen synthase and glycogen branching enzyme (*3*). The finding that an endurance-training program is associated with GDE activity increases along with that of phosphorylase indicates that the ratio of phosphorylase to debranching enzyme activity must be regulated in order for complete glycogen degradation.

A deficiency in the debranching enzyme complex underlies glycogen storage disease type III (Cori's Disease) (*134*). The lack of activity of GDE results in an incomplete breakdown of glycogen and glycogen accumulates. The glycogen stored in the tissues affected by the disease has very short outer chains.

The clinical presentation of GSD-III is remarkably diverse (*135*). Most patients present with deficiency of the enzyme in liver and muscle and the disease is characterized by liver enlargement, hypoglycemia, and shortness in stature (*136*). In about 15% of patients, the disease appears to only involve the liver. Liver-related symptoms typically improve with age and resolve after puberty (*137*). Muscle weakness can occur in patients with a deficiency of GDE in muscle, with the severity of the weakness typically progressing in severity from very mild in childhood to severe after the third or fourth decade of life (*138-140*). The diversity of symptoms observed in GSD-III patients is most likely due to the differences in tissue expression of the deficient

enzyme. Patients having both muscular symptoms and liver involvement seem to suffer from a generalized enzyme defect. In these patients, deficient enzyme activity also occurs in tissues such as the heart, erythrocytes, and cultured fibroblasts (*141, 142*). Patients have also been reported to have only liver involvement with no clinical or laboratory evidence of myopathy (*136*). There have been rare cases where selective loss of either the glucosidase activity or the transferase activity of the bifunctional has been demonstrated (*135, 141*).

While the regulation of GDE remains obscure, the study of the enzyme in clinical medicine has demonstrated that the enzyme is important in the proper metabolism of glycogen. Also, the one study done by Taylor and colleagues did report that the enzyme does increase its activity in response to an endurance training program (*4*). The body of work on this enzyme supports the idea that the branching and debranching complexes of the glycogen cycle enzymes are important and that organisms relying on glycogen for metabolic energy regulate the ratios of the enzymes that are active in the processes of branching and debranching of glycogen. Despite this evidence, the relative paucity of investigation into both the branching and debranching enzymes is apparent, especially in comparison to the relatively large body of work done on glycogen synthase and, to a lesser extent, with glycogen phosphorylase. Further work into the regulation of glycogen branching and debranching enzymes would be desirable.

Implications for Future Research

In the decades since the discovery of glycogen phosphorylase in 1939, the study of glycogen and its metabolism has changed the fields of biology and clinical medicine. Discoveries of such regulatory mechanisms as reversible phosphorylation, allosteric effectors, the cyclic

AMP signal cascade, and hormonal control were all intimately related to investigations of glycogen synthesis and degradation.

Despite so many decades of intense research effort, many gaps in our understanding of the regulation of glycogen metabolism still exist. Furthermore, there remains vigorous debate in such areas as whether or not glycogen exists as one species within the cell or as distinct metabolic entities clearly distinguishable by characteristics such as acid solubility and protein content.

An area of critical importance in determining the structure and metabolic regulation of glycogen is the branching/debranching system. The fact that the key distinguishing characteristic separating glycogen from starch is the greater degree of branching found in glycogen is one point demonstrating the importance of branching. The lethality of glycogen storage disease type IV, caused by a deficiency of the branching enzyme, further underscores the significance of branching. Moreover, a deficiency of debranching enzyme causes glycogen storage disease type III, which has a milder clinical course and a greater diversity in clinical presentation than GSD-IV, but nonetheless results in symptoms ranging from cirrhosis of the liver to cardiomyopathy in particularly severe cases. Aside from the glycogen storage diseases, it is also worth noting that a high degree of branching results in the formation of more terminal glucose residues incorporated into the mature glycogen granule. Both glycogen synthase and phosphorylase have their catalytic activity at the terminal residues, therefore the rate of both glycogen synthesis and breakdown would be accelerated by more branching.

Despite these points, the roles played by branching and debranching enzyme in the regulation of glycogen synthesis and degradation have received almost no attention in exercise

science. There have been a total of two studies performed on both enzymes examining their relevance to exercise; both were published in the early 1970's and the results have not stimulated any further interest, despite providing evidence that both enzymes increase following exercise training. Many questions remain open. Do the two enzymes differ in their concentrations in fast versus slow-twitch muscle? Since fast-twitch muscle is adapted to perform high-intensity exercise, it is reasonable to suggest that glycogen is a preferred fuel in fast-twitch muscle when compared to slow-twitch muscle. A finding of different concentrations of branching and de-branching enzyme in the two different muscle types would be a novel finding that would add to the current state of knowledge in glycogen enzymology.

The liver is also of interest as a site of possible differing concentrations of branching and de-branching enzyme. It could be expected to find higher concentrations of the two enzymes in the livers of animals that perform high-intensity, glycogen-depleting exercise due to the key role played by liver glycogen in buffering blood glucose levels during times of scarcity and exercise. However, we currently do not know whether or not this is true. Further questions of interest regarding the branching and de-branching system in the liver include determining whether or not diet has an effect. Dietary manipulation could exert an effect on glycogen branching structure independent of exercise by influencing the rate of flux through glycogen and thereby imposing a metabolic stressor on the enzymatic systems controlling the structure of glycogen. The potentially differing effects of a high-carbohydrate versus a low-carbohydrate diet on glycogen branching in the liver, is a topic that invites inquiry. Currently the scientific literature is silent on this subject.

We also do not know if glycogen branching and de-branching enzymes vary in concentration in skeletal muscle among different individuals according to their exercise-training

status. Two animal models are particularly attractive as candidates for investigation that could answer this question. Racehorses and dogs both represent a class of elite animal athletes that could provide a standard of comparison with their non-elite counterparts. Data suggesting a difference between the two groups could be extended to other species, including humans, which might open new vistas in understanding how glycogen responds to exercise.

Glycogen branching and de-branching enzymes are essential to life in mammalian species. Clinical medicine has established that deficiencies in these enzymes are lethal. However, despite the vast amount of inquiry directed towards glycogen synthase and phosphorylase in regulating glycogen in clinical and exercise contexts, branching and de-branching enzymes have eluded investigation. The significance of these enzymes likely extends beyond the question of whether they are present or not. Glycogen is a molecule that is subject to elaborate regulation, representing a major investment of energy in the organism. Given the importance of glycogen as a nexus in bioenergetics and the essentiality of the branched structure of glycogen, the question of whether or not the enzymes controlling the branching and de-branching of glycogen are subject to modulation in response to exercise and diet is one that merits study.

CHAPTER II – EXPERIMENTS

Introduction

The significance of the glycogen branching and debranching enzymatic systems in metabolism is made plain by the fact that a deficiency of either enzyme results in disease. In the case of a deficiency of branching enzyme, the clinical course of the disease is fatal, while a deficiency of debranching enzyme results in clinical outcomes varying in severity. Indeed, the single structural feature of glycogen that distinguishes it from plant starch is the greater degree of branching found in glycogen.

Furthermore, the branching of glycogen makes glycogen more soluble in the cell and modifies the structure of the polysaccharide so as to create a greater number of non-reducing ends, which serve as a substrate for phosphorylase. This structural characteristic of branched glycogen allows for a more rapid degradation of glycogen in times of high metabolic demand and is thus adaptive for the organism.

In spite of the importance of branching in glycogen metabolism, there has been a relative lack of scientific interest in the study of branching enzyme when compared with the voluminous body of literature that has developed surrounding enzymes such as glycogen synthase and glycogen phosphorylase. Although these enzymes are thought to be rate limiting in the biosynthetic and degradative arms respectively, of glycogen metabolism, there has been some evidence that branching enzyme is regulated in response to stimuli.

Although preliminary, the few studies examining branching enzyme activity in experimental trials have lent support to the idea that branching enzyme is regulated in response to genetic modification and exercise. The only study to investigate branching enzyme's response

to exercise was published in 1974 (*3*). Using human subjects, Taylor and colleagues reported that branching enzyme activity increased in response to exercise training. The method used to assay branching enzyme in this experiment was not made clear, however. The observed increase in branching enzyme activity reported in the 1974 study was related to an earlier study by the same authors (*143*) reporting that glycogen synthase activity increased in response to exercise training in parallel with branching enzyme. The implication of this relationship was that the activities of glycogen synthase and glycogen branching enzyme are regulated in response to exercise such that the ratios of the two enzymes remains intact and that the length of the α 1,4-glycosidic chain remains constant, along with the number of non-reducing ends in the glycogen macromolecule.

Although the method of measuring the activity of branching enzyme was not clear in the 1974 study, a subsequent study by Pederson and colleagues (*117*) using genetically modified mice provided evidence showing a relationship between overexpression of glycogen synthase and branching enzyme. In this study, mice overexpressing constitutively active glycogen synthase in skeletal muscle were shown to have elevated muscle glycogen and a decreased degree of branching. However, Western blotting showed that despite the decreased degree of branching observed, there was an increase in the level of branching enzyme expression and an approximately threefold increase in branching enzyme activity. Branching enzyme was assayed according to the method developed by Krisman (*144*), which uses an iodine staining reagent to determine the degree of branching colorimetrically using a spectrophotometer.

The development of an assay for branching enzyme activity has posed challenges. Methods devised have included a periodate oxidation assay for end-group determination, which can provide the basis for a calculation of the average chain length of the branches in a polysaccharide and therefore a measure of the degree of branching (*145*). Another method

involves the treatment of amylopectin (starch) with branching enzyme and determination of the degree of branching caused by the addition of branching enzyme by the decrease in absorbance of a colored complex of the polysaccharide and iodine reagent (*144*). Recognizing that the structural difference between glycogen and amylopectin is the degree of branching of each, it was noted that the two polysaccharides have different wavelengths of maximum absorption when stained with iodine and analyzed with a spectrophotometer. Amylopectin has a wavelength of maximum absorption of 520 nm while that of glycogen is 460 nm. The principle of the iodine-staining assay is to take aliquots of a reaction mixture containing branching enzyme and iodine-stained amylopectin at different time intervals and determine the decrease in absorbance of the colored complex at 520 nm. As the polysaccharide becomes more branched, absorbance at 520 nm decreases.

While this iodine method is specific for the activity of branching enzyme, it is neither quantitative nor sensitive. The method cannot give a specific quantity of branch points formed by the action of branching enzyme but can only determine that the degree of branching is more or less as indicated by the change in the color of an indicator. The method is also subject to interference from impurities in branching enzyme preparations or in the polysaccharide itself. Enzyme preparations and glycogen from tissue extracts are frequently contaminated with amylase, an enzyme that degrades polysaccharides by the hydrolysis of α-1,4-glycosidic bonds. The presence of this enzyme interferes with the measurement of branching enzyme by the iodine method because it degrades the polysaccharide at the same time it is being synthesized by the reaction mixture containing specific quantities of branching enzyme. While the assay can be corrected for amylase interference, the procedure is time-consuming and still does not overcome

the fact that the assay cannot directly quantify the number of α-1,6 branch points synthesized by branching enzyme specifically.

An attempt was made by Krisman and colleagues to develop an assay for branching enzyme that is sensitive, specific, and quantitative (*108*). The basic procedure of the assay is to first synthesize a polysaccharide using a reaction mixture containing a small amount of glycogen as a primer with other reaction components necessary for the synthesis of complete macromolecular glycogen. The amount of purified branching enzyme added to the reaction mixture was varied and aliquots of the polysaccharide formed were taken and analyzed at intervals. The polysaccharide synthesized as described above was then purified and subjected to complete degradation by the combined actions of phosphorylase and debranching enzyme. The principle of the assay is to selectively quantify the glucose residues involved in branch points catalyzed by the action of the branching enzyme and express the degree of branching of the polysaccharide by comparing the number of the glucose residues bound to the polysaccharide by α-1,6-glycosidic linkages as a percentage of the total carbohydrate content of the polysaccharide. This is possible due to the differing products yielded by the catalytic actions of phosphorylase and debranching enzyme.

In the degradation step mentioned above, the combined actions of phosphorylase and debranching enzyme are both necessary to break down glycogen, or any branched polysaccharide, completely. In the breakdown of glycogen specifically, it is significant that debranching enzyme yields free glucose by the hydrolysis of α-1,6-glycosidic bonds while the product of the phosphorylase reaction is the phosphorylated sugar glucose 1-phosphate. The separate actions of the two enzymes are diagrammed below.

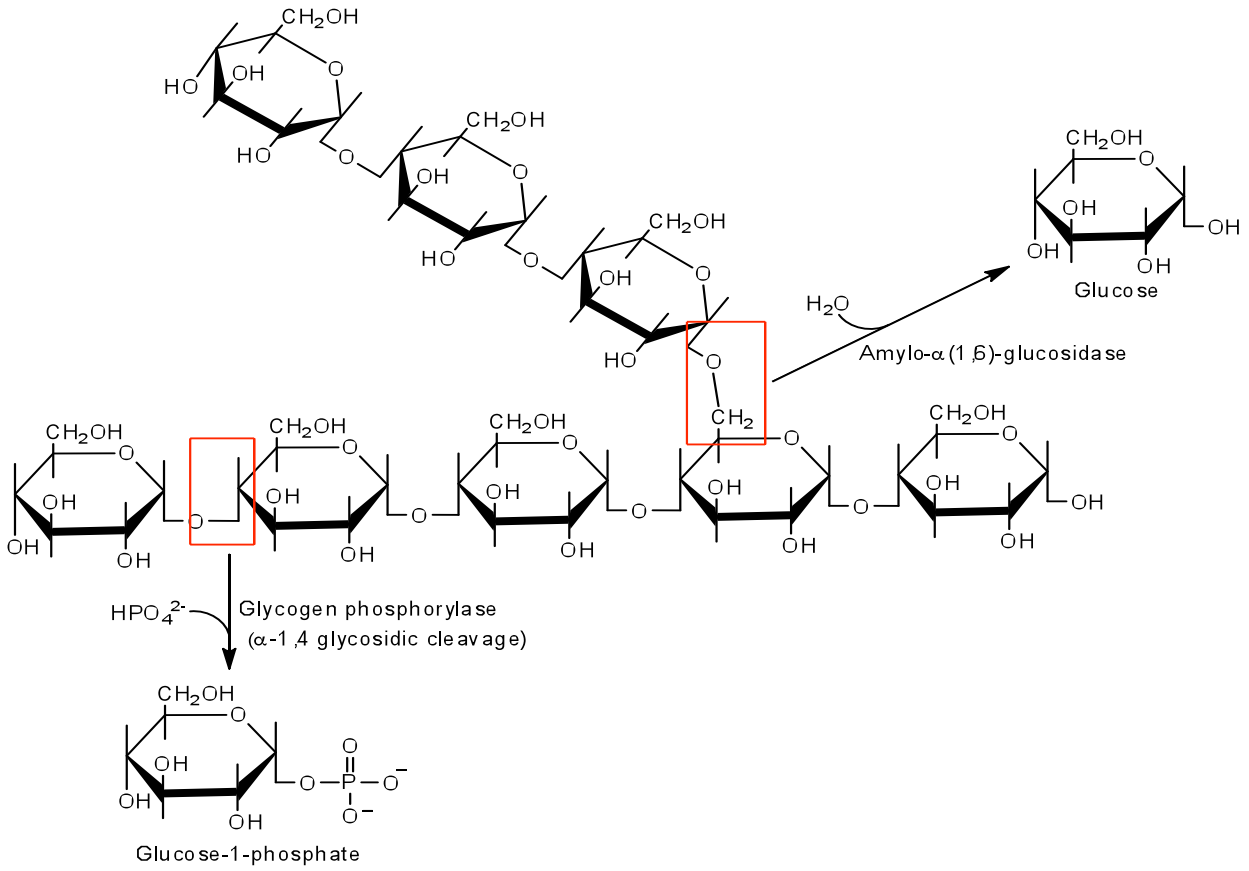

Figure 2.1. The separate actions of amylo-α(1,6)-glucosidase and glycogen phosphorylase yield the different products, free glucose and glucose-1 phosphate, which are determined separately and compared using the assay developed by Krisman and colleagues.

The two products formed by the reactions depicted above allow for the quantitative determination of the branch points because the free glucose yielded by the hydrolysis of the α-1,4-glycosidic linkages is a reducing sugar while the glucose 1-phosphate yielded by the phosphorolysis of the α-1,4-glycosidic bonds is not a reducing sugar. Therefore, the measurement of reducing sugars in a sample obtained by the breakdown of purified glycogen is a measurement of the free glucose that can have come from no other origin but the α-1,6-linkages formed by the action of the branching enzyme. In turn, the quantity of the reducing sugar

measured by an assay that is specific to the presence of such sugars can be compared to the quantity of total carbohydrate in the sample to give a percent degree of branching.

The advantages of the assay are several. First, it is quantitative due to the fact that it measures directly the product formed by branching enzyme. The specificity of the assay is also desirable since a number of reducing sugar assays are available that are sensitive only to reducing sugars and no other carbohydrates. Krisman and colleagues also demonstrated in their study describing the development of the assay that the method shows a strict linear correlation with the number of glucose units linked by branch points and not the overall amount of polysaccharide synthesized in the reaction. This linear relationship holds even when the branching enzyme used in the reaction is known to be contaminated with amylase; the method therefore overcomes a common problem in measuring branching enzyme. This allows the method to be used in crude extracts in which amylase interference would otherwise be a problem in determining branching enzyme activity. Krisman and co-workers also indicated that the method is more sensitive than another quantitative assay developed decades ago, the periodate oxidation assay, which requires 25 times more polysaccharide for determination of the number of α-1,6-glucosyl residues than does the method described here. The periodate method also depends on the degree of branching of the polysaccharide under study, with lower degrees of branching requiring greater amounts of sample in order for the assay to detect the degree of branching (*108*). The assay is also inexpensive in terms of reagents and equipment.

Since the development of Krisman's method for the selective measurement of glucose residues from the branch points in a polysaccharide, the assay has been surprisingly little used when considering its advantages relative to other techniques. This should not be interpreted to mean that the assay is not valid, but rather is more likely an indication of the relative lack of

scientific interest in studying branching enzyme when compared to other highly active areas of research pertaining to glycogen, such as glycogenin, glycogen synthase regulation, and the pro- and macro-glycogen debate. Regardless of the cause, only a few studies were undertaken to extend the results of the initial work done in developing the assay (*146-148*). These studies applied the assay to brain tissue and added insight into possible regulation of the enzyme, along with revealing more detail about the structure of glycogen. However, no attempt has been made to use the assay to develop a body of data on the degree of glycogen branching in a variety of species and tissues, nor has there been any application of the method in a clinical trial in which diet or exercise has been employed as an intervention to determine if branching enzyme is modulated in response to such stimuli. In recognition of the fact that only one study sought to examine the role of exercise in modulating branching enzyme (*3*), and that this particular study was unclear regarding the method used to assay the enzyme, there appears to be a gap in our understanding of branching enzyme from several vantage points.

One area of interest would be to provide data from several samples of the same type of tissue in an animal model to get a more complete idea of the normative range of the degree of branching in tissues. Such data is completely lacking at the current time and would add to our understanding how glycogen structure might be different across a range of tissues and species. A particular area to investigate is whether or not the degree of branching of glycogen varies in tissues that are specialized for particular functions, such as fast twitch versus slow twitch muscle or in liver compared to muscle in general.

Dietary manipulation is also an intervention that could shed additional light on how branching enzyme might vary in response to stimuli and possibly exert a heretofore unrecognized regulatory role in glycogen synthesis. While such a role for branching enzyme is

speculative, there is a rationale for an up-regulation of branching enzyme being a facilitator in repletion of glycogen stores following depleting exercise or fasting. The additional solubility gained by incorporating more branch points into the structure of glycogen, along with the more ready availability of branched glycogen as a substrate for glycogen synthase and phosphorylase lend theoretical support to the idea that branching enzyme might be regulated. Data from a study with genetically modified mice indicates clearly that branching enzyme expression is increased when the animals overexpress an active form of glycogen synthase (*117*). The data from this particular investigation provides an intriguing set of data indicating a regulatory role for branching enzyme, but it is important to note that the method used to assay the enzyme was the iodine staining method (*144*), which is less sensitive than the method described above and is also not quantitative.

The branching enzyme assay developed by Krisman might also be modified so as to increase its economy and simplicity. The reducing sugar assay method employed to quantify the number of glucose residues involved in branch points is that developed in the 1930's by Somogyi (*149*). This colorimetric assay is sensitive and specific but requires intensive effort in the preparation of several reagents and is more time consuming and labor intensive than some other methods introduced in the many years since its original development. One such assay is the tetrazolium blue assay (*150*), developed in 1984. This assay uses a colorless, water-soluble tetrazolium salt as an indicator for the detection of reducing sugars in a sample. The tetrazolium reagent undergoes transformation to a water-insoluble formazan salt, which develops a blue color when heated for 30 seconds in the presence of a reducing sugar such as glucose. This colored compound is extracted from the aqueous component of the reaction mixture by the addition of toluene and then the absorbance is read by a spectrophotometer at 570 nm. The

glucose concentration is then interpolated from a standard calibration curve. A detailed description of the assay principle and procedures follows in another section but the assay is more economical in terms of reagents and time than the Somogyi method while remaining sensitive and specific.

The basic method developed by Krisman will be applied to a cohort of mice subjected to a dietary intervention. This study will aim to clarify the degree of glycogen branching in the liver tissue of an animal in which the enzyme has not yet been measured. From the application of this method, a set of new normative data will be developed which will help to clarify the structure of glycogen in the mouse liver. Furthermore, the experiments described below will seek to determine if a dietary intervention brings about a modulation in the activity of branching enzyme in mice.

Methods

Experimental Animals

The laboratory animals and dietary and quercetin supplementation procedures described here were developed and implemented in their entirety by Henagan and colleagues (*151*). Liver tissue specimens were a generous gift of Dr. Laura Stewart.

The Male C57BL/6J mice (n = 240) (Jackson Laboratory, Bar Harbor, ME) were obtained and weaned onto a high carbohydrate diet (Research Diets, New Brunswick, NJ). The high carbohydrate diet had a fat content of 10% of kcals from fat. At 6 weeks of age, the mice were randomly assigned in equal numbers to one of the following diets, the fat content of each diet is indicated according to percent of kcals in the diet from fat: 1. High carbohydrate (HC) (10% kcals fat), 2. Low carbohydrate (LC) (45% kcals fat), 3. Low carbohydrate + quercetin (LC

+ Q500) (45% kcals fat + 500 µg/mouse/day), 4. Low carbohydrate + quercetin (LC + Q250) (45% kcals from fat + 250 µg/mouse/day), 5. Low carbohydrate + quercetin (LC + Q50) (45% kcals fat + 50 µg/mouse/day). The groups are summarized in the table (Table 2.1) below.

Table 2.1 Summary of experimental diet and supplementation protocols

Treatment Group	Fat Content of Diet (% of kcals in diet)	Quercetin Dose (µg/mouse/day)	Number of Animals in Group
HC	10%	-	48
LC	45%	-	48
LC + Q500	45%	500	48
LC + Q250	45%	250	48
LC + Q50	45%	50	48

Mice were quartered individually in shoebox cages with corncob bedding. Temperature was controlled (22°C). The light: dark cycle was maintained at 12 : 12 hours. All experiments were reviewed and approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

Diets and Food Consumption

The procedures described in this section pertaining to diets and food consumption were developed and implemented in their entirety by Henagan and colleagues.

Quercetin ≥ 98% (HPLC) was purchased from Sigma Aldrich (St. Louis, MO) and LC + Q diets were stored at 4°C in light-protected, airtight containers. Food was changed every 3 days and water was provided *ad libitum*. Food consumption was measured on a weekly basis in 16 representative mice week 0 to week 4 and from 8 representative mice from week 4 to week 8 in

each treatment group. Food consumption was measured by weighing food before and after a 48 hour period each week.

Isolation of Liver Glycogen

At 3 weeks and 8 weeks, mice were euthanized and immediately post-mortem the livers from the experimental animals were harvested and immediately snap frozen in liquid nitrogen. After freezing, the tissues were stored at -80°C until ready for further analysis.

The basic steps in isolating liver glycogen consist of digestion of the tissue in hot concentrated alkali, in this case potassium hydroxide (KOH), followed by precipitation of the glycogen with ethanol (*152*). The detailed procedure is outlined below (*145*).

A sample of liver tissue was dropped into a previously weighed glass test tube containing 3 ml of 33% KOH solution. The tube was then weighed once again and the weight of the sample determined by the difference (n = 138, 265.1 mg ± 110 mg). The tube was then immersed in a boiling water bath for 20 to 30 minutes. Following tissue digestion, the tube was vortexed briefly and 0.5 ml of saturated sodium sulfate was added. The glycogen was then precipitated by the addition of 4 ml of 100% ethanol. This mixture was then vortexed briefly before the tube was then reheated until the mixture began to boil. The tube was then removed from heat and cooled on ice for 15 minutes. Following cooling, the mixture was centrifuged for 15 minutes at 3000 rpm. The alcoholic supernatant was then decanted and the test tube allowed to drain. The precipitate was then dissolved in 2 ml of distilled water and frozen at -80°C until ready for further analysis.

Purification of Glycogen Debranching Enzyme

Debranching enzyme was not routinely available commercially and was therefore partially purified from dog muscle until the 41% ammonium sulfate fractionation step as described by Brown and Brown (*153*).

The details of the purification procedure are described below. Dog muscle was obtained by a generous donation of the Calcasieu Parish Animal Shelter in Lake Charles, Louisiana. Animals from which muscle samples were collected were euthanized by phenobarbital injection. Immediately post-mortem muscle samples were dissected from the quadriceps and triceps brachii and frozen at -20°C until ready for further processing.

The frozen dog muscle was kept overnight at 6°C so that it partially thawed in preparation for homogenization. The homogenization procedure was carried out in a cold room at 6°C. The partially frozen muscle samples, weighing approximately 1 gram each, were placed in a glass test tube in 3 ml of cold distilled water and the cells disrupted by a Brinkmann Polytron PT 10/35 homogenizer (Brinkmann, Switzerland). Following cell lysis, the crude extract was centrifuged (Sorvall) at 23,000 g for 1 hour at 4°C. The precipitate was discarded and the supernatant was adjusted to pH 6 with 2 *M* sodium acetate buffer, pH 4.5. The supernatant was then centrifuged again at 10,000 g for 15 minutes at 4°C and the precipitate was discarded. The supernatant was then neutralized to pH 7.0.

After adjusting to pH 7.0, the neutralized solution of 450 ml initial volume was made 41% saturated in ammonium sulfate by the slow addition of 108 g of solid ammonium sulfate as the solution was stirred. The solution was allowed to stand at 4°C for 2 hours, after which time the precipitate was collected by centrifugation at 10,000 g for 15 minutes at 4°C. The supernatant

was discarded and the precipitate was suspended in 5mM Tris-1 mM EDTA-5 mM β-mercaptoethanol, pH 7.2. This solution was then dialyzed for several hours against frequent changes of the buffer. Following this step, the precipitate was collected by centrifugation at 10,000 g for 15 minutes at 4°C. The precipitate was re-suspended in buffer and then frozen in aliquots at -80°C for later use. The protein concentration of the preparation was determined by Bradford Assay to be 1.09 µg/µl.

Tetrazolium Blue Assay for Reducing Sugars

The tetrazolium blue assay was originally developed as a sensitive reducing sugar assay to measure the cellulolytic activity in bacterial cultures. The method is based on the formation of a colored formazan salt when colorless tetrazolium salt is reduced in the presence of a reducing sugar such as D-glucose, first observed by Cheronis (*154*).

The colored formazan salt is water insoluble and has a wavelength of maximum absorption of 570 nm. The assay allows for simple determination of reducing sugars with a spectrophotometer. The assay used in this investigation takes advantage of a modification introduced by Mullings and Parish (*150*), which adds a toluene extraction step. The addition of the toluene extraction step uses the solubility of the colored formazan salt in organic solvent to overcome problems encountered in quantifying the reducing sugars colorimetrically in the aqueous layer caused by the particulate nature of the substrate. The method is sensitive and specific. It is also simple and economical in terms of reagents when compared to other reducing sugar assay methods, such as the Nelson-Somogyi assay (*155*). The details of the assay procedure are given below.

The tetrazolium blue reagent was prepared by adding three volumes of 0.3 *M* NaOH to an aqueous suspension of tetrazolium blue chloride (1% w/v, 1 volume) (Sigma Aldrich, St. Louis, MO) and stirring until completely dissolved. Five volumes of distilled water were then added and the reagent stored at 5°C in the dark. For each assay 0.1 ml of sample and 0.9 ml of tetrazolium reagent were mixed in a tube and then immersed in a boiling water bath for 30 seconds. The tubes were allowed to cool to room temperature and then 1.5 ml of toluene was added to each tube. Each tube was then shaken using a vortexer. The shaking was repeated until no more color was removed from the aqueous layer. 1 ml of the toluene layer was then placed into a quartz cuvette by pipette and absorbance at 570 nm was recorded. Reducing sugar concentration in the sample was quantified from interpolation from a standard curve with D-glucose as the reference standard. The calibration curve for D-glucose, using least squares analysis to give the best fit to points, is shown (Figure 2.2) below.

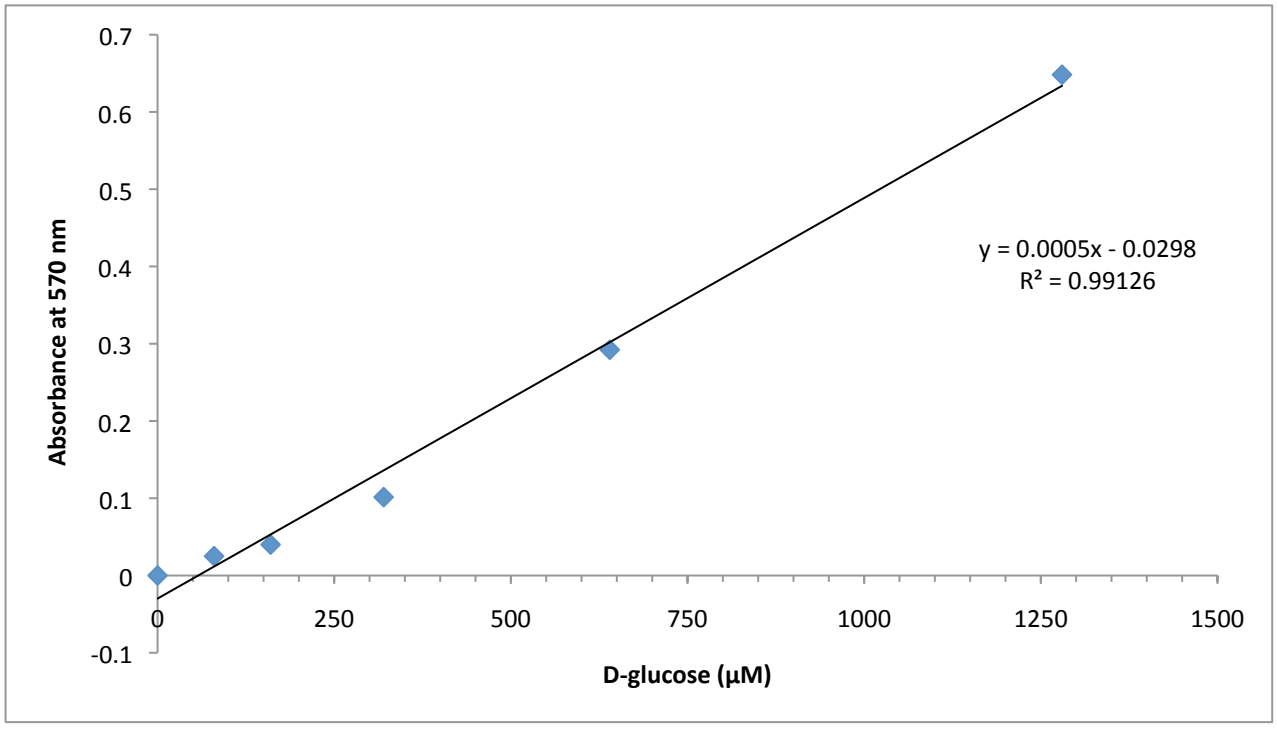

Figure 2.2. Calibration curve for the tetrazolium blue assay for reducing sugars

The assay demonstrated sensitivity to a lower detection limit of 80 μM of D-glucose. The calibration curve was calculated using the mean value of five replicates for each D-glucose concentration. The intraclass correlation coefficient was 0.99.

Phenol Sulfuric Acid Assay

The phenol sulfuric acid assay for total carbohydrate is one of several assays based on the action of concentrated sulfuric acid that causes the hydrolysis of glycosidic linkage. The hydrolyzed neutral sugars (pentoses and hexoses) are then partially dehydrated, with the elimination of three molecules of water, to form furfural or a derivative of furfural.

Furfural and its derivative cause condensation with a number of several phenolic compounds such as phenol (*156*), α-naphthol, orcinol (*157*), and anthrone (*158*) to form colored compounds (*159*). The particular phenolic compound used in this experiment was α-naphthol (Molisch's reagent), which reacts positively with all carbohydrates and forms a purple colored product with a wavelength of maximum absorption of 490 nm. Details of the procedure are below.

Table 2.2. Various colorimetric assays for sugars based on phenolic compounds (adapted from reference 159)

Name of Test	Phenol	Concentrated Mineral Acid	Positive Reaction	Color of Product
Molisch's	α-naphthol	H_2SO_4	All carbohydrates	Purple
Seliwanoff's	Resorcinol	HCl	Ketoses and sucroses	Red
Bial's	Orcinol	HCl	Pentoses and uronic acids	Green
Tollen's	α-naphthol	HCl	Uronic acids	Blue

Molisch's reagent is prepared by making a 5% w/v solution of α-naphthol in 100% ethanol. 200 µl of the reagent is added to a glass test tube and mixed with 200 µl of glycogen sample. For this experiment, three replicates of 200 µl each were taken from the glycogen isolated from the liver of each animal. 1 ml of concentrated sulfuric acid was then added rapidly and directly on the sample without allowing the acid to touch the side of the tube. The solution was left undisturbed for 10 minutes and was then vortexed. Following vortexing, the solution was incubated for another 30 minutes. 1 ml was then taken from each tube and then placed in a fresh polystyrene cuvette. Each sample was then read by a spectrophotometer (Shimadzu UV-160) at 490 nm and the carbohydrate concentration determined from a standard plot. The standard calibration curve was calculated using D-glucose as the reference. Five replicates were used for each concentration and the mean of the replicates for each concentration used as the standard. The calibration curve is shown below (Figure 2.3.)

The samples developed color with an optical density that exceeded the linear working range of the assay. Therefore each sample was diluted so that the absorbance was within the working range of the assay and the concentration was then determined by interpolation from the standard curve, taking the dilution factor of each sample into account when making the calculation. The calibration curve showed linearity within a working range from 40 µM up to 1.28 mM. The lower limit of detection observed during development of the standard plot was 40 µM. The ICC for the assay calibration curve was 0.998.

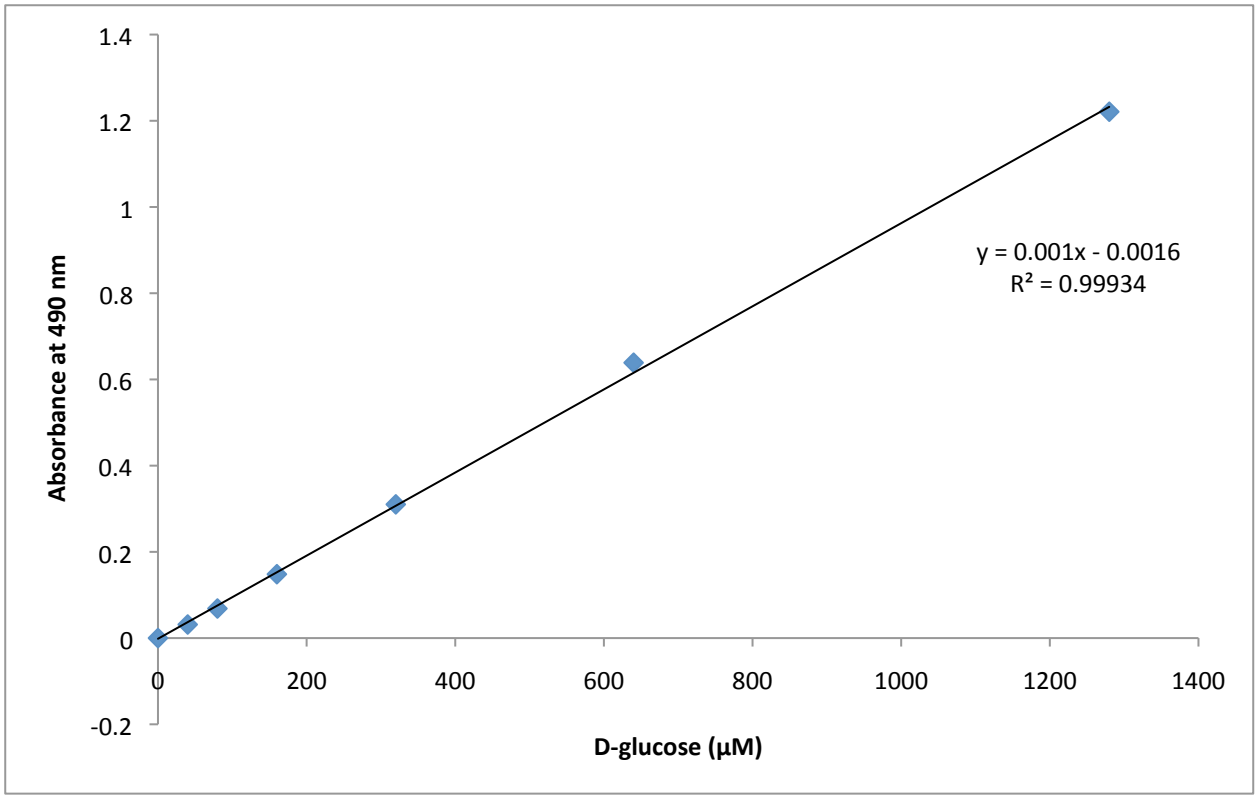

Figure 2.3. Calibration curve for the phenol sulfuric acid assay for total carbohydrate

Preparation of Reagents for the Determination of the Degree of Branching

Purified AMP (Sigma A2252) was obtained by purchase from Sigma Aldrich (St. Louis, MO). The AMP was solubilized in cold distilled water and ammonium hydroxide, which was added drop-wise to the cold stirring mixture of AMP and distilled water until the AMP was completely dissolved. The solution was then adjusted to pH 7.0 and frozen in aliquots at -80°C until ready for use. As noted in the section entitled *purification of glycogen debranching enzyme,* debranching enzyme was not available commercially and was therefore purified from dog muscle up to the 41% ammonium sulfate fractionation step by the procedure of Brown and Brown. Phosphorylase b (Sigma P6635) was purchased from Sigma Aldrich (St. Louis, MO) and was solubilized in 0.6 *M* potassium phosphate buffer, pH 7.0. The solution was frozen in aliquots to eliminate potential problems caused by freeze-thaw cycles at -80°C until ready for further use.

Determination of Degree of Branching of Mouse Liver Glycogen

To determine the degree of branching in the mouse liver glycogen samples under study, a modification of the procedure developed by Krisman (*108*) was used. This procedure has the advantages of being sensitive, specific, and quantitative. It is also economical in terms of equipment and reagents and is well suited to measurement of branching enzyme activity in crude extracts due to the observation by Krisman and colleagues that the method overcame interference by amylase-type degrading enzymes (*108*). The specific measurement of branching enzyme activity in mouse liver was carried out by the following procedure described below:

A. Purification of glycogen from mouse liver.

Mouse liver tissue extracts had been kept frozen at -80°C and glycogen was purified from the tissue by the methods described in detail in the section above entitled *isolation of liver glycogen*. Briefly, the glycogen samples were placed in 3vol of 33% KOH and heated in a water bath at 100°C for 20-30 minutes, until complete digestion. 4 ml of 100% ethanol and 0.5 ml of saturated sodium sulfate was added to the digest to precipitate the glycogen. Following centrifugation for 15 minutes at 3000 rpm, the precipitate was collected and the supernatant was discarded. The precipitate was solubilized in 2 ml of distilled water.

B. Measurement

To determine the number of glucose residues involved in the branch points the glycogen was degraded by the coordinate action of phosphorylase b and debranching enzyme. The reaction mixture contained 200 µl of the purified glycogen, 60 mM potassium buffer with 21.66 µl of phosphorylase b (Sigma P6635, Sigma Aldrich, St. Louis, MO) dissolved in the buffer, pH 7.0, 5 mM AMP (Sigma A2252, Sigma Aldrich, St. Louis, MO) and the debranching enzyme

preparation purified from dog muscle (0.85 mg of protein, as determined by the Bradford Assay). The reaction mixture was brought up to a final volume of 1300 µl by the addition of distilled water. The reaction mixture is summarized in the table (Table 2.3) below.

Table 2.3. Summary of reaction mixture for degradation of glycogen purified from mouse liver

Reaction Component	Concentration in Stock Solution	Concentration in Reaction Mixture	Volume of Stock Solution Added to Reaction Mixture (µl)
Potassium phosphate buffer with dissolved Sigma P6635 phosphorylase b, pH 7.0	600 mM 1 µg/6 µl	60 mM 21.66 µg	130
AMP (Sigma)	50 mM	5 mM	130
Debranching Enzyme	1.09 µg/µl	0.85 mg of protein	780
Glycogen from Mouse Liver	Unknown Sample	Unknown Sample	200
Distilled H_2O	-	-	60
Final Volume			1300 µl

The reaction mixture was combined in a 2.0 ml microfuge tube and incubated for 3 hours at 37°C in a water bath. Following incubation, the reaction was stopped by the addition of 3 vol of 100% ethanol. After centrifugation at 200 rpm for 15 minutes in a micro-centrifuge (Eppendorf, Germany), a precipitate layer was observed at the meniscus slightly above the 400 µl mark of the tube. Two aliquots of 100 µl were taken from the bottom layer of the mixture, below the precipitate layer, and added to glass test tubes for measurement of reducing sugar by the tetrazolium blue assay.

The glucose measured in these aliquots was then compared to the total carbohydrate in separate aliquots of the purified glycogen isolated from the same liver by the phenol sulfuric acid assay. The degree of branching is expressed as the ratio between the number of glucosyl residues

linked by α 1,6-glycosidic linkages measured by the tetrazolium blue assay (*150*) and the total carbohydrate determined by the phenol sulfuric acid method (*159*).

Experiment 1

Experimental Approach to the Problem

The development of an assay for glycogen branching enzyme has faced challenges over several decades. One of the first experimental approaches to the problem was the periodate oxidation method, which gives a measurement of the number of non-reducing end glucose units in the glycogen macromolecule (*160*). The principle of the assay is based on the observation that when six-carbon sugars are attacked by periodate the ring structure is disrupted with the removal of the third carbon atom as formic acid. After periodate oxidation of glycogen, the quantitative determination of the formic acid produced by the reaction allows for a calculation of the average chain length of the branches in the glycogen. This method, although in general agreement with other methods (*108, 161*), is time consuming and depends on the color change of an indicator. Accurate determination of the degree of branching also is also dependent on the structure of the polysaccharide under analysis, with polysaccharides with low branch content requiring up to 25 times the amount of polysaccharide required when compared to the method developed by Krisman (*108*).

Another method developed to assay the activity of branching enzyme is the iodine-staining method of Krisman (*144*). This method works by incubating amylopectin with branching enzyme and measuring the decrease in optical density at 520 nm, the wavelength of maximum absorption of amylopectin. As catalysis by branching enzyme proceeds, the absorbance of the iodine-stained product at 520 nm continues to decrease and the wavelength of

maximum absorption shifts toward 460 nm, the wavelength of maximum absorption of glycogen. This method is specific and rapid. However it is essentially non-parametric and therefore cannot provide a quantitative determination of the activity of branching enzyme and rather can only give a relatively greater or lesser degree of activity of branching enzyme as the optical density of the polysaccharide changes as the degree of branching changes.

Krisman and colleagues developed a method to measure the activity of branching enzyme by selective quantitation of the α-1,6-linked glucose residues involved in the branch points (*108*). This assay is sensitive, specific and quantitative. The assay synthesizes a polysaccharide using known quantities of purified glycogen branching enzyme and then completely degrades the synthesized polysaccharide. The number of α-1,6-linked glucose residues are measured in the reaction mixture as reducing sugars and this is compared to the amount of total carbohydrate determined by the phenol sulfuric acid assay (*156*) to yield a percent degree of branching. The determination of the number of glucose residues involved in the branch points by measuring reducing sugars in the reaction mixture of degraded glucose is valid due to the fact that free glucose, which is a reducing sugar, can come from no other origin than a branch point. This free glucose is a product of the action of debranching enzyme, which hydrolyzes α-1,6-glycosidic linkages selectively. All other carbohydrate degraded in the reaction mixture is glucose-1 phosphate, which is liberated by the action of phosphorylase and is not a reducing sugar. The assay method therefore compares metabolically distinct carbohydrates from known origins in the glycogen macromolecule and expresses a percentage of free glucose as a constituent of the entire glycogen molecule.

The method of Krisman and colleagues has only been applied (*146-148*) in a few experiments. These studies have not extended the application of the method to a clinical trial, nor

has the method been used to assay branching enzyme activity in any particular animal model in repeated trials that could build up a set of data that would allow conclusions about norms of branching enzyme activity to be developed. The reliability and reproducibility of the method has also not been tested. Moreover, while the method is simpler and less time consuming than the periodate oxidation method in particular, it is noteworthy that the reducing sugar assay employed by Krisman is the Somogyi-Nelson method (*149*). This method was developed in 1933 and, despite its sensitivity and specificity, might be a less attractive method for the determination of reducing sugars than some alternative procedures developed in the decades since its inception. One such assay is the tetrazolium blue assay, developed in 1984 by Mullings and Parish (*150*). This method is very economical in terms of reagents and provides results in less than an hour. The assay is sensitive and specific to reducing sugars.

In this experiment, the method of Krisman and colleagues will be used to measure the degree of branching in the liver glycogen from mice. This is the first experiment performed using this assay method in mouse liver. The sample size used in the experiment is larger than in any study previously done using the method. The reliability and reproducibility of the assay will also be tested for the first time. Also, the use of the tetrazolium blue assay for reducing sugars instead of the Somogyi-Nelson method is a new adaptation of the original method used by Krisman and is simpler, more economical, and less time consuming than the Somogyi-Nelson method.

Methods

The experimental procedures for the experiment are outlined in detail above. Briefly, liver samples (n = 68) from mice were collected and analyzed for the degree of branching of glycogen isolated from each animal. To assay the degree of branching, the method of Krisman

(*108*) was used, with the modification that the tetrazolium blue assay for reducing sugars was used instead of the Somogyi-Nelson method.

The degree of branching is reported as a percentage based on the ratio of reducing sugars assayed by the tetrazolium blue assay to the total carbohydrate content measured by the phenol sulfuric acid assay (*156*).

Statistical Analysis

A major aim of the study is to test the reliability of the assay method. Therefore, optical density of the samples assayed by the tetrazolium blue and phenol sulfuric acid assays were measured in duplicate and triplicate, respectively. The reducing sugar and total carbohydrate content were determined from the mean optical density of the replicates of each assay and were determined mathematically by interpolation from a standard calibration curve developed in our laboratory using known glucose concentrations as the standards. The calibration curve for each assay was developed using five replicates for each glucose concentration with the mean optical density of the replicates as the value for each concentration of glucose. The optical density was measured using a Shimadzu UV160-U UV-VIS spectrophotometer (Shimadzu, Columbia, MD).

To test the reliability of the assays, intraclass correlation coefficients (ICC) were determined for the replicates of the tetrazolium blue assay and the phenol sulfuric acid assays; ICC values were obtained for each assay both for the calibration curves and the measurement of carbohydrate content in the liver samples. Degree of branching for all 68 mice was measured and the results reported as mean ± standard deviation. All statistical procedures were completed using SPSS version 20 (Chicago, IL).

Results

The assay procedures demonstrated a high degree of reliability for each of the assay conditions, measuring glucose concentrations for the development of standard plots and measuring the carbohydrate content of the liver samples. Table 3.1 below shows the ICC values for each assay. The mean degree of branching for the 68 mice under study was 13.14 ± 8.71 percent.

Table 2.4. Intraclass correlation coefficients for the tetrazolium blue and phenol sulfuric acid assays

Assay and Condition	ICC
Phenol – H_2SO_4 for Liver Samples	0.925
Phenol – H_2SO_4 for Standard Calibration Curve	0.998
Tetrazolium Blue for Liver Samples	0.848
Tetrazolium Blue for Calibration Curve	0.99

Calibration curves developed for each assay calculated from known concentrations of D-glucose are shown in figures 2.5 and 2.6 below.

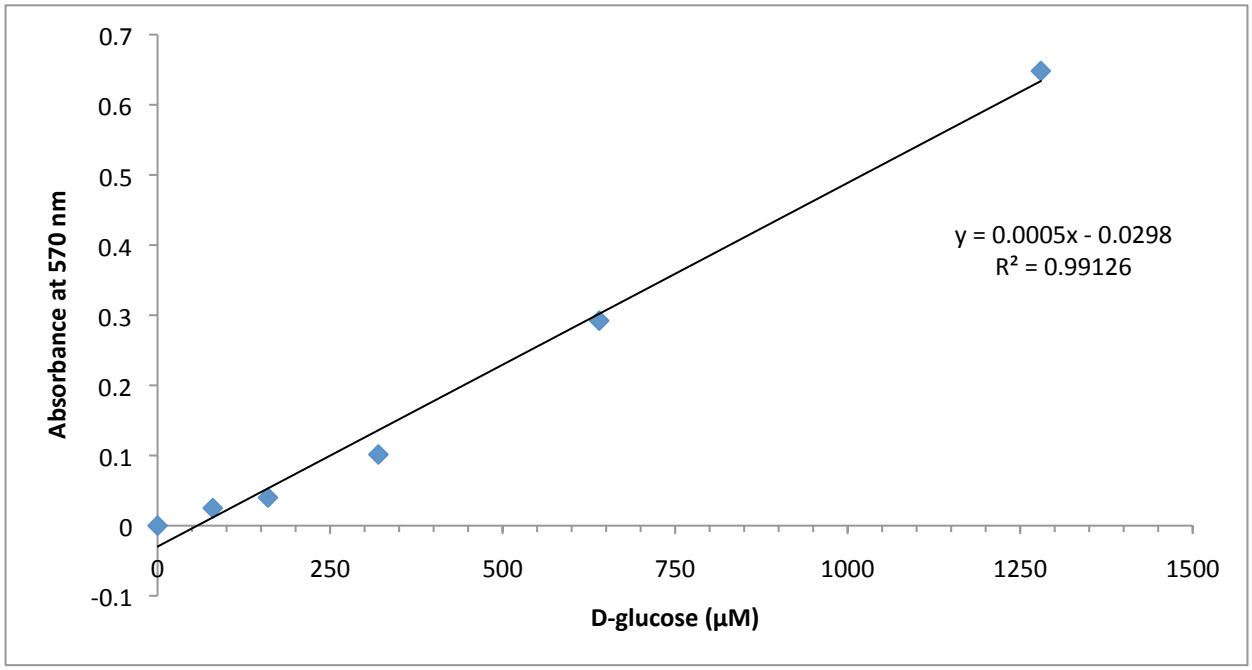

Figure 2.4. Calibration curve for the tetrazolium blue assay

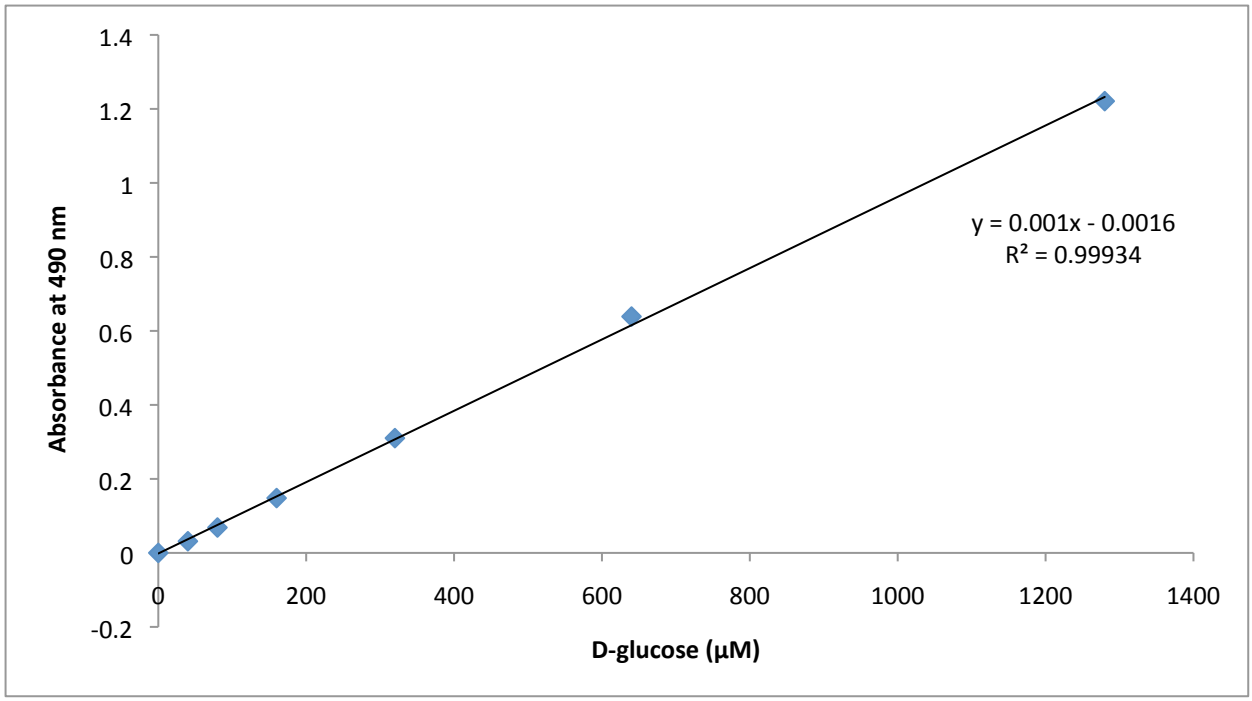

Figure 2.5. Calibration curve for the phenol sulfuric acid assay

Discussion

The aims of the present study were mainly to apply the branching enzyme assay developed by Krisman and extend its application to a new animal model. This experiment also used a large sample size in an effort to determine the degree of branching of glycogen in mouse liver and also made an initial effort to assess the reliability of the assay. The values for the degree of branching found in the current study (13.14 ± 8.71%) are in approximate concordance with values reported by Krisman and Tolmasky (9.4% for rabbit liver glycogen) (*108*). The degree of branching reported in this study is also roughly similar to that reported in a standard biochemistry textbook (*119*), which was expressed as "about 1 in 10 residues is branched." For direct comparison, 13% degree of branching would correspond to a ratio of 1 in 7.7 residues being branched. It is important to note, however, that the extent of agreement with the previous work done using this assay principle is difficult to report with certainty with our current state of

knowledge. Previous work done using the method did not report results with any indication of the variance found in the degree of glycogen branching among the animal and plant specimens assayed. It is unclear from the previous work how many different animal samples were tested since the authors did not report the degree of branching in terms of means with measures of variance. Instead, the values were reported singly for each plant or animal tissue under study (*108, 148*).

This experiment also used a simpler and more economical reducing sugar assay than the Nelson-Somogyi method, which was employed in the original development of the assay. Along with the effort to establish a framework of normative data within which to view the degree of branching of mouse liver glycogen, data has been presented on the reliability of the assay method. This report also makes clear the degree of reliability and linearity of the standard plots used to quantify reducing sugars and total carbohydrate in the liver samples; an accurate measure of these quantities depends entirely on the accuracy of the calibration curves used to interpolate these values. The results reported here are intended to facilitate comparisons of various protocols to assay glycogen branching enzyme and thereby advance the aim of accurately measuring the enzyme in a way in which valid comparisons of the activity of the enzyme can be made across different tissues and species. At the current time, this is not possible due to a lack of comparable data in the literature.

The results of the statistical analyses performed in the present study indicate that the assay procedure demonstrates a high degree of reliability. The intraclass correlation coefficients for the tetrazolium blue assay for reducing sugars and the phenol sulfuric acid assay for total carbohydrate when used to develop standard calibration curves and to measure carbohydrates in the samples provide evidence that the assay methods generate optical density values that are

stable when measured with multiple replicates (ICC's were 0.99, 0.848, 0.998, and 0.925 respectively). It is also important to note that the numbers of replicates for each tetrazolium and phenol sulfuric acid assay are clearly reported in this study in an effort to enable the protocols described here to be repeated independently without confusion.

One of the difficulties in developing the assay used in the present study was the lack of information providing any indication of the actual levels of reducing sugars or total carbohydrate one could expect to find in the samples under study. This complicated efforts to develop the calibration curves for D-glucose upon which accurate measurement of reducing sugars and total carbohydrate depend. The process of developing calibration curves with the reliability and linearity required for accurate measurement of carbohydrates in the samples required many repetitions of the assays before standard plots were generated that captured the optical densities measured in the tissue samples under study. Even after extremely high linearity ($R^2 = 0.9913$ for the tetrazolium assay and $R^2 = 0.9993$ for the phenol sulfuric acid assay) was attained for the calibration curves across a wide range of glucose concentrations (40 μM – 1.28 mM), it was necessary to perform dilutions and back calculations in order to measure the total carbohydrate by the phenol sulfuric acid assay due to the extremely high optical density in the undiluted samples. The dilution factors necessary for obtaining optical density readings within the working range of the assay ranged from 4X to 50X. By including the calibration curves and making clear that dilutions were required we hope to facilitate efforts to replicate the procedures here with a view toward refinement of the method.

The decision to use the tetrazolium blue assay to measure reducing sugars in the present study was made in order to increase the economy of the assay procedure in terms of cost, simplicity, and time required to collect data. The assay also showed a high degree of sensitivity

(*150*) when first reported in the literature. The reducing sugar assay developed by Somogyi in 1933 (*149*) has been used for several decades and is time-honored for its sensitivity and reliability but it requires more reagents and is more complex than the tetrazolium assay. These characteristics provide an incentive to adopt a simpler method, which the tetrazolium blue assay offers. The assay was quite new at the time Krisman and Tolmasky first reported their method and it is possible that during the time these authors were developing their procedure for measuring branching enzyme they were as yet unaware of the assay.

The high degree of reliability of the tetrazolium assay reported here should offer encouragement for researchers to further test the assay in terms of its applicability to measuring branching enzyme activity. The sensitivity of the tetrazolium assay measured in our laboratory was comparable to that reported by Mullings and Parish in the original report describing the procedure, with linearity in our standard curve found at a lower limit of 80 μM of D-glucose compared to 100 μM reported by Mullings and Parish (*150*). Achieving reliable results with the tetrazolium assay might require some repetition for those inexperienced in its use. In samples with only monosaccharides, the heating time of 30 seconds can probably be exceeded without any threat to validity. This is due to the fact that extended heating time will cause alkaline hydrolysis of oligosaccharides, which can cause inaccuracies when the intent of the procedure is to measure only reducing sugars such as glucose. While we endeavored to be precise in keeping the heating time at 30 seconds, any deviation from that standard did not necessarily threaten the accuracy of our results since any alkaline hydrolysis of pure glycogen would result in the release of glucose, which was the sugar we intended to measure.

Another inherent source of variability in measuring reducing sugars using the tetrazolium assay involves the toluene extraction step, which takes place after heating the reaction mixture.

The procedure involves repeated shaking of the reaction mixture, which is composed of an aqueous layer and a toluene layer, which are distinct due to the fact that toluene and water are not miscible. The formazan salt formed by the reduction of tetrazolium blue in the presence of a reducing agent such as glucose is insoluble in water and highly soluble in toluene. However, repeated agitation of the sample is required in order for the toluene to completely extract the colored product. Repetition of the assay is required in order to accurately determine when the color is completely extracted into the toluene layer, which is above the aqueous layer due to its relatively low density compared to water. It is worth noting that the toluene extraction step was an improvement introduced by Mullings and Parish when they developed the assay based on the initial observation by Cheronis (*154*) that tetrazolium could be used to quantify reducing sugars. Observations in our laboratory confirm that the assay overcomes the problem of the particulate nature of the formazan salt when it is confined to the aqueous layer. When the colored formazan salt is suspended in the toluene layer, the high degree of solubility of the formazan salt in toluene leads to the formation of a very clear solution with no particulate matter. Measurement of the optical density of the colored product in the toluene layer is thus more accurate than it would be in the aqueous layer due to the particulate nature of the substrate in aqueous solution.

An important aim of the present study was to employ a large sample size of glycogen specimens in an effort not only to test the reliability of the assay with multiple iterations but to supply data which could be used to draw some conclusions about norms of branching enzyme in the mice under study. Moreover, the testing of the degree of branching enzyme in a large set of subjects offers some data about the variability of the degree of glycogen branching across animals of the same species in the same tissue, mouse liver in this case. Also, the report here of a mean value of the degree of branching in mouse liver allows a side by side comparison with

values found for other tissues and species reported by Krisman and colleagues (*146, 148*). These authors found degrees of branching of 9.4%, 10.7%, and 7.9% in glycogen from rabbit liver, rat brain, and oyster, respectively. The reports of Krisman and colleagues do not contain data on samples sizes used nor do they report the degree of branching as means ± standard deviation. It is therefore impossible to draw any conclusions from these reports on the variability of branching enzyme activity with respect to different tissues and species.

In the present study we find that the average degree of branching of mouse liver glycogen in the 68 animals studied to be 13.14 ± 8.71 percent. This is the first study to our knowledge to report on a sampling of subjects of this size and the first to report average values of the degree of branching in the same type of tissue from so many different animals of the same species. On the basis of this, there is at least some preliminary evidence that the degree of branching in mouse liver exhibits a rather high degree of variability across individual animals. When examined together considering the data reported here on the reliability of the assay, it seems fair to conclude that the degree of branching in the glycogen samples under study was accurate at least in terms of accurate measurement of the reducing sugars and total carbohydrate, which are the variables necessary to measure in order to ascertain a degree of branching. It is necessary to consider other steps in the procedure that could have introduced uncontrolled variability into the measurement, however.

The isolation of the glycogen from each liver sample is a procedure that involves steps that can cause variability from one tissue extract to the next. While the digestion of the tissue extracts in hot alkali is a time-honored practice that denatures glycogen-degrading proteins and is generally not subject to variability across different extracts, the ethanol precipitations and re-suspensions of the glycogen pellet after centrifugation are steps that could vary in technique

across samples and investigators. The decision of how many ethanol precipitations to perform before the glycogen is suspended in water is not entirely clear-cut and therefore could create variability. The amount of time between the extraction of the liver tissue from the mice and the snap-freezing in liquid-nitrogen also could affect the glycogen concentration in the liver samples. Glycogen degrading enzymes remain active at temperatures down to -60°C and therefore it is possible that some of the glycogen from the samples could have been broken down prior to snap-freezing if the samples were not frozen in liquid nitrogen immediately upon dissection. If some of the glycogen from the tissue samples was lost due to post-mortem degradation, the degree of branching might have been affected due to the activities of glycogen phosphorylase and de-branching enzyme. It is difficult to say exactly how the degree of branching would be affected by such glycogen breakdown, with the understanding that different relative activities of phosphorylase and de-branching enzyme would alter the degree of branching in opposite directions if either enzyme predominated.

The degree of branching could also be affected by how complete the degradation of the glycogen was when subjected to the action of phosphorylase b and de-branching enzyme in the reaction mixture designed to degrade the glycogen in preparation for the tetrazolium assay. The incubation time and reaction mixture were in exact accordance with the procedures detailed by Krisman and Tolmasky. The authors noted that the reaction conditions used in their assay method resulted in near complete degradation of the glycogen into free glucose and glucose-1 phosphate, as determined by radioactive tracer studies on ^{14}-Cglucose used to synthesize the polysaccharide that was subsequently degraded using the same procedure as in the present study. This study replicated the reaction procedure used by Krisman and Tolmasky to the highest degree of exactitude possible. The reagents were purchased from Sigma and were therefore

reasonably assured to be of the highest purity attainable. The only exception to this was the de-branching enzyme preparation, which was not available commercially and therefore was isolated from dog muscle according to the procedure of Brown and Brown (*153*), which was the same method employed by Krisman and Tolmasky to obtain de-branching enzyme in their original study.

The procedure for the purification of the de-branching enzyme from muscle extracts is a rather lengthy one when taken to completion, but for the assay of branching enzyme, it is only necessary to proceed to the 41% ammonium sulfate fractionation step in order to replicate the assay procedure of Krisman and Tolmasky. It is important to note here that the assay does not require a minimum level of purity as such, but rather it requires a certain level of protein content (0.85 mg). This requirement was fulfilled in our assay procedure as the Bradford assay (*162*) for total protein content was used to determine with precision the protein content of the de-branching enzyme preparation purified from dog muscle in our laboratory. The amount of de-branching enzyme added to the reaction mixture was determined so that exactly 0.85 mg of total protein was introduced into the mixture. All other reaction components were adjusted to accommodate this amount of de-branching enzyme preparation while keeping the molarity of all the other components in exact agreement with the procedure of Krisman and Tolmaksy.

As an additional control step to verify that the de-branching enzyme was active, a reaction mixture containing a blank replacing the de-branching enzyme preparation with distilled water was used as a comparison. The mixture was identical to the one outlined by Krisman and Tolmasky with the exception that the de-branching enzyme was not present. The reaction mixture containing the blank was negative for reducing sugar when tested with the tetrazolium assay. The reaction mixture containing the de-branching enzyme tested positive for reducing

sugar, confirming that the de-branching enzyme preparation from our laboratory was active. We were therefore certain to an acceptable degree that the actions of phosphorylase and de-branching enzyme were complete and the glycogen from each sample was completely degraded.

The main objectives of the present study were to extend the applications of the branching enzyme assay developed by Krisman and Tolmasky to a large sample size of mouse liver samples in an effort to gain normative data about branching enzyme activity and to gain information that would allow some quantification of the reliability of the assay method. To this end we reported a high degree of reliability of the assay method not only in measuring the degree of branching in the samples but also in the development of the calibration curves used to quantify reducing sugars and total carbohydrate, which are the two variables required to determine the degree of branching. The number of replicates for each test was clearly reported to facilitate repetition of our procedures. The average degree of branching of a large number of subjects was reported that gives some normative data about the degree of branching of mouse liver glycogen and the variability of the measurement across different samples of the same tissue within one species. The degree of branching can thus be compared to the values reported by Krisman and Tolmasky with a view toward expanding the data available for estimating norms for glycogen branching enzyme across species and tissues. Finally, the assay method of Krisman and Tolmasky was modified with the intent of simplifying and economizing the procedures by using the rapid and economical tetrazolium blue assay for reducing sugars rather than the Somogyi-Nelson method.

Experiment 2

Experimental Approach to the Problem

The branched structure of glycogen is critical to proper metabolic function in animals. Absence of glycogen branching enzyme is an inherited error of metabolism resulting in Glycogen Storage Disease Type IV (GSD IV), which in humans is fatal, usually by age 2 (*163*). While the clinical significance of an inherited deficiency in glycogen branching enzyme has been generally recognized by researchers active in the study of carbohydrate metabolism, there has been comparatively little interest in studying whether branching enzyme activity and/or concentration differs in subjects not suffering from GSD IV. Rather, much of the research effort directed toward studying branching enzyme has aimed at developing and refining clinical testing procedures for GSD IV (*164*).

The body of scientific inquiry into the regulation and modulation of branching enzyme activity is scant. One study by Taylor and colleagues investigated the response of branching enzyme to an exercise training regimen in human subjects (*3*). The study found that branching enzyme activity increases in response to chronic exercise but the method used to assay branching enzyme was not made clear in the study. Pederson and colleagues found that in mice overexpressing constitutively active glycogen synthase there was a concomitant increase in branching enzyme expression and activity, but not of a magnitude sufficient to prevent the accumulation of abnormal glycogen in skeletal muscle, which precipitated and acted as a foreign body in the tissue (*117*). Interestingly, the livers of the transgenic mice did not appear to be affected. This study, however, used the iodine staining method of Krisman (*144*) to assay the activity of branching enzyme. This method uses the long-established observation that the degree of glycogen branching influences the absorption spectrum of glycogen intercalated with iodine.

The method, however, is essentially non-parametric and therefore cannot provide a quantitative determination of branching enzyme activity, although the method is specific and rapid.

Krisman and Tolmasky developed an assay method that is specific, sensitive, and quantitive (*108*). The method selectively quantifies the glucose residues from α-1,6-linkages from the branch points and compares the number of such glucose residues to those from total carbohydrate content of the same glycogen sample. This value is then expressed as the percentage degree of branching in the glycogen particle and is a parametric value that can be compared quantitatively to values obtained by the same method across any range of branched polysaccharide samples.

The method of Krisman and Tolmasky could be a significant improvement in the measurement of glycogen branching enzyme. However, the method has not been employed extensively. In particular, it would be of interest to use the method to explore whether branching enzyme increases in response to dietary intervention, since dietary intake modulates the rate of glycogen synthesis and concentration in tissues. The present study aims to determine whether or the relative percentages of carbohydrate and fat in the diets of mice has an effect on the degree of branching of mouse liver glycogen. The method of Krisman and Tolmasky will be used to determine the degree of branching, with a modification of the protocol that simplifies the assay and is less expensive in reagents and is less time consuming (*150*).

Methods

A detailed account of the experimental procedures has been given above. Briefly, mice were fed a high carbohydrate (n = 29) and low carbohydrate diet (n = 39) and were euthanized at 3 weeks and 8 weeks. Liver samples from all the animals were assayed for branching enzyme

activity using selective measurement of the glucose residues involved in the branch points expressed as a percentage of the total carbohydrate content of the glycogen sample under study.

Statistical Analysis

The degree of branching of the glycogen samples from the LC- and HC-fed mice livers were compared using a t-test. Glycogen concentration of the liver samples of the two groups was also compared. The level of statistical significance was set *a priori* at $P \leq 0.05$. Statistical testing was performed using SPSS version 20 (Chicago, IL).

Results

Analysis of the data did not show a statistically significant difference in the degree of branching between the LC- and HC-fed mice ($P = 0.87$). The degree of branching between the two groups actually showed a surprisingly high level of agreement (LC $\bar{x} = 13.06 \pm 8.68$, HC $\bar{x} = 13.41 \pm 8.68$). The difference in glycogen concentration was also found not to be statistically significant ($P = 0.28$).

Table 2.5. Comparison of Degree of Branching and Glycogen Concentration - values are mean ± SD.

	High Carbohydrate	Low Carbohydrate
Percent Degree of Branching	13.41 ± 8.68	13.06 ± 8.68
Glycogen Concentration (μmol)	2476.6 ± 1633.5	3080.7 ± 2998.6

Discussion

The results of the test of degree of branching in LC- and HC-fed mouse livers did not support the hypothesis that modifying the diets of the animals would result in a change in the

activity of branching enzyme between the two groups. An additional finding was that the difference in glycogen concentration between the two groups was not statistically significant.

It might be expected that a high carbohydrate, and consequently high carbohydrate, might promote greater flux through glycogen and therefore would be stimulatory to the enzymes governing the rates of glycogen biosynthesis. The literature abounds with evidence that high carbohydrate intake promotes a higher rate of glycogen synthesis than that observed with a low carbohydrate diet, particularly when replenishment of depleted glycogen stores following strenuous exercise is studied.

This study is the first to apply the assay method developed by Krisman and Tolmasky to a large group of subjects in a clinical intervention. Previous work in our laboratory has provided evidence that the assay procedure shows a high degree of reliability and it was therefore of great interest to apply the technique in an interventional study design to determine whether there were differences in branching enzyme brought about by different diets. The data did not reveal significant differences in the degree of branching of glycogen between the two groups. However, it is interesting to note a few points.

The relative agreement in the measurement between the two groups adds to the body of data gathered thus far in evaluating the assay method and provides an enhanced frame of reference for comparison to values found in other species and tissues in the original study published that made the assay method known (*108*). The present study found that the degree of branching of the liver samples of both groups was approximately 13 percent. This value is not far from the value reported for rabbit liver glycogen (9.4 percent) in the original study of Krisman

and Tolmasky. Other samples measured in that study include oyster glycogen (7.9 percent), amylopectin (5.7 percent), and the limit dextrin of rabbit liver glycogen (15.6 percent).

While the values reported by Krisman and Tolmasky do give a framework in which to place the values found in the current study, it is noteworthy that the data provided by Krisman and Tolmasky in the two studies in which they employ their method (*108, 148*) do not specify the sample sizes used in their protocol, nor is there any data reported on variance in their study. In the present study, a high degree of variance was observed in the degree of branching in both experimental groups. This could indicate that branching enzyme activity is more heterogeneous across individual animals than the results of previous work might lead one to conclude. In fact, throughout the rather scant body of literature reporting on branching enzyme, there are no studies that use sample sizes as large as that in the current study and no study has been encountered thus far that reports on the variability found in measurements of the enzyme.

Of additional interest is the observation that the glycogen concentrations found in the liver samples of the two groups did not differ significantly. It is difficult to account for this finding in light of the evidence that a high carbohydrate diet tends to promote higher glycogen storage than does a low carbohydrate diet. In an effort to use the largest sample sizes possible for the study, mice that were euthanized at different times in their life span and at different times of day were pooled into two groups with respect only to the types of diet they consumed. Had a significant difference between the groups been observed, such a finding would have been very robust given the relative heterogeneity of the two groups in attributes other than diet.

The relationship between glycogen concentration and the degree of branching has been of interest in some studies of the relative activities of glycogen synthase and branching enzyme. For

example, Pederson and coworkers found that mice overexpressing constitutively active glycogen synthase demonstrated a concomitant increase in branching enzyme activity and expression (*117*). The increase in branching enzyme was of insufficient magnitude to prevent the accumulation of abnormal glycogen that precipitated in skeletal muscle, but not in liver. In the present study, it was of interest to determine if any relationship between glycogen concentration and degree of branching would be observed. If there had been a higher number of branch points found in parallel with a higher concentration of glycogen, a reasonable explanation for the finding would be that the ratio of elongation activity of glycogen synthase is matched and varies positively with the activity of branching enzyme. This would have been in agreement with the findings of previous work (*3, 117, 143*). However, the findings of the current study indicated that the degree of branching was very similar among HC- and LC-fed mice and likewise the glycogen concentration did not differ significantly between the groups.

This investigation into whether or not a dietary intervention is associated with a change in the degree of glycogen branching did not support the hypothesis that such a relationship exists. Results of this study indicate that the degree of branching of mouse liver glycogen is similar, although slightly higher, to that reported by Krisman and colleagues for rabbit liver glycogen and rat brain (*108, 148*). The study also did not observe any significant differences in liver glycogen concentrations between mice fed a low carbohydrate/low carbohydrate versus a high carbohydrate/high carbohydrate diet.

Experiment 3

Experimental Approach to the Problem

The second of the three experiments detailed here explored the possibility of a relationship between diet and the degree of branching in mouse liver. The third experiment will examine whether or not the dietary supplement quercetin, in conjunction with different diets, has a measureable effect on the degree of branching in the mouse liver.

Quercetin is a flavonoid found widely in nature. A body of evidence has emerged indicating that quercetin might have beneficial effects on some biological parameters related to health. Anti-inflammatory properties have been reported (*165*). A positive effect has also been observed on mitochondrial biogenesis (*166*). However, there is some evidence that quercetin can aggravate insulin resistance in liver brought about by a low-carbohydrate diet (*167*).

The development of insulin resistance in liver is of interest as it pertains to the metabolism of glycogen. One of the most important functions of the liver is to synthesize glycogen and export glucose liberated from its own glycogen stores to other tissues in times of high metabolic demand. Insulin promotes an increase in the rate of glycogen biosynthesis in the liver and the development of insulin resistance is an impediment to the proper functioning of the liver as a repository of glycogen.

It would be of interest to determine if metabolic perturbations to the liver brought about by insulin resistance would have a modulatory effect on glycogen structure in the liver. To this end, the present study aims to test whether different dietary intakes, in conjunction with different dosing regimens of quercetin, affect the degree of branching in the liver glycogen of mice subjected to such treatment. The degree of glycogen branching might well be negatively influenced by a decreased flux of glucose into hepatocytes, which would be observed if insulin

resistance developed in the liver. To address this question, several different dosages of quercetin were administered within the same mouse model to determine its effect on liver glycogen structure as it pertains to the degree of branching of liver glycogen.

Methods

A detailed account of the procedures used in the experiment is given above in the extended methods section previously in the document. A brief summary of the methods follows below.

Mice were weaned onto a high carbohydrate diet for 6 weeks and were then randomly assigned in equal numbers to the experimental groups summarized in the table below.

Table 2.6. Summary of the diets and quercetin dosing regimens of the subjects

Treatment Group	Fat Content of Diet (% of kcals in diet)	Quercetin Dose (µg/mouse/day)	Number of Animals in Group
HC	10%	-	48
LC	45%	-	48
LC + Q500	45%	500	48
LC + Q250	45%	250	48
LC + Q50	45%	50	48

At 3 weeks and 8 weeks, mice were euthanized and liver samples were harvested at the zenith (n=8) and the nadir (n=8) of the metabolic cycle and immediately snap-frozen in liquid nitrogen for measurement of the degree of glycogen branching by the procedure detailed in the extended methods section above.

Statistical Analysis

The data were analyzed with SPSS version 20 (Chicago, IL) and the results are expressed as means ± standard deviation. Measurements were evaluated using a one-way ANOVA. If significance was detected between the means by ANOVA, a post-hoc test was used to determine which groups were significantly different from one another. In this case, the assumption of homogeneity of variances between the different groups was not fulfilled. Therefore the Welch and Brown-Forsythe robust tests for equality of means were used to test for statistical significance. The Games-Howell post-hoc test was used to determine which groups were significantly different from each other. The threshold for statistical significance was set at $P < 0.05$ *a priori*.

Results

A statistically significant difference ($p = 0.042$) was observed between the low-carbohydrate diet group supplemented with 500 µg of quercetin that was euthanized at night at 8 weeks versus the low-carbohydrate diet group supplemented with 500 µg quercetin euthanized during the day at 8 weeks. No other statistically significant differences were observed.

Table 2.7. Percent degree of branching - values are means ± SD. (* denotes $p < 0.05$)

Treatment Group	Degree of Branching
8W Day LC 500	7.27 ± 2.69*
8W Night LC 500	21.16 ± 6.99*
8W Night LC	9.71 ± 7.98
3W Day LC 50	13.20 ± 10.17
8W Day LC 50	16.75 ± 8.47
3W Day HC	13.48 ± 10.84
3W Night HC	7.83 ± 7.35
8W Day HC	14.52 ± 8.24
8W Night HC	17.42 ± 6.19

Discussion

The only interaction between the treatments and the degree of branching appeared to be between the groups supplemented with 500 μg/day and euthanized at 8 weeks. The only difference between the two groups was the time in the metabolic cycle during which they were euthanized. Descriptive statistics for all the mice cohorts are compiled and tabulated in the appendix (pp.106 – 108).

A possible explanation for this observation is that the one group was replete with liver glycogen while the other was depleted. Mice are typically most active at night and therefore feed at that time. It would therefore be expected that at the end of the dark cycle that the liver glycogen of the animals would be at its peak. However, there were no significant differences in liver glycogen concentrations observed between any of the experimental groups. It would therefore be highly speculative to propose an explanation of for the cause of this difference between these two groups.

It is essential to note that the high variance in the degree of liver glycogen branching observed in all groups of mice in this experiment. It is also important to recognize that each cohort of mice was no larger than 8 individuals. In some cases, due to errors in measurement there was attrition in some groups that resulted in there being fewer than 8 individual mice in a group. Combined with the high degree of variance observed in the degree of branching, the relatively small sample size of each group undoubtedly limited the power of the study to detect any differences between the groups.

This is the first study to apply a quantitative assay of branching enzyme to a prospective cohort study involving dietary intervention and a supplement regime. Previous experiments using

this procedure were intended mainly to test the method and to use the assay to learn more about the biochemical mechanisms involved in glycogen branching rather than to assess whether or not the enzyme is regulated or changes in response to various metabolic stimuli.

While the results of the current study do not provide convincing evidence that glycogen branching enzyme activity is modulated in response to diet or quercetin supplementation in mouse liver, the assay method does offer some potential in measuring the enzyme in other contexts in which the experimental design is optimized to reveal changes in the activity of the enzyme.

Possible areas of interest might include extending the use of the assay to different tissues such as different fiber types of skeletal muscle, which differ in metabolic profile and glycogen content. Given the increasing prevalence of metabolic syndrome and diabetes, a protocol that measures the enzyme's activity in diabetic versus healthy animals with a large sample size might reveal differences in the degree of glycogen branching that accompany perturbations in carbohydrate metabolism in diabetes.

SUMMARY

The study of glycogen has led to discoveries of general significance in biochemistry and clinical medicine. Since the discovery of the glycogen molecule by Claude Bernard in 1870 to the pioneering discoveries of Carl and Gerty Cori, whose efforts identified glycogen phosphorylase, the first allosteric protein ever studied, the study of glycogen has revealed new vistas that have led to advances applicable to all areas of biological science. Following upon the trail blazed by the Coris, Luis Leloir discovered the enzyme that catalyzed the key step in glycogen synthesis, glycogen synthase. Not only did work of Leloir provide the key to understanding the synthesis of glycogen, but his experiments also led to an understanding of the essential role played by nucleotide sugars, which not only are activated donors in glycogen synthesis but key structural elements in DNA and RNA.

Of critical importance in glycogen structure is the branching of the molecule. The only difference in the chemical structures of plant starch and animal glycogen is the different degree of branching in each polysaccharide, with glycogen being more branched than its plant counterpart, amylopectin. The branched structure of glycogen is critical to proper metabolic function in animals. The absence of glycogen branching enzyme, which is inborn error of metabolism, results in glycogen storage disease type IV, which is invariably fatal in humans, usually by age 2.

Given the crucial importance of glycogen branching in metabolism, it would be reasonable to conclude that this feature of glycogen structure would draw significant scientific interest. In a way, that statement is true. In clinical medicine, tests for branching enzyme have been developed that can differentiate whether or not the enzyme is completely absent. This is of obvious significance in diagnosing disease, but the study of branching enzyme has languished in

comparison with the massive effort directed at understanding the regulation of glycogen synthase and phosphorylase.

As a consequence of this relative lack of interest in glycogen branching enzyme, there have been relatively few efforts to develop assay methods to measure the activity of the enzyme. The most frequently employed assay procedure, to the extent that the enzyme has been studied at all, was a procedure developed in 1962 to measure the enzyme based on the observation that the degree of branching of glycogen influences the absorption spectrum of glycogen intercalated with iodine. This method is not parametric, however, and therefore does not measure the enzyme quantitatively. This makes it impossible to compare values obtained from different tissues and animal specimens.

The development of a quantitative method for the assay of glycogen branching enzyme took a further two decades following the iodine staining assay. In 1985, Krisman and Tolmasky published a study reporting their development of an assay procedure that was sensitive, specific, and quantitative. The method directly measured the degree of branching by accurately and selectively measuring the glucose residues linked to the glycogen molecule by α-1,6-glycosidic linkages and expressing the degree of branching as the ratio of these glucose residues as a percentage of total carbohydrate. The development of this assay offers a way of measuring branching enzyme that allows numerical comparisons of the degree of branching across species and tissues.

Up to the current time, this assay has not been utilized to give an idea of the degree of branching in the glycogen of different animals and tissues. Therefore, we still have no concept of norms of glycogen branching, whether we are discussing different species or tissues. The method

also allows determination of variability in glycogen branching among various tissues and species. The series of experiments detailed in this document took up the task of applying this assay method to liver samples of mice, an experimental model that had not been examined before. Other key objectives included measuring the reliability of the assay and examining whether or not dietary intervention and nutritional supplementation resulted in any modulation of the enzyme. The assay was also modified to economize the use of reagents and reduce the time necessary for the collection of data.

The first experiment measured the degree of glycogen branching in the largest sample of subjects yet assembled for the study of the enzyme. Detailed data were reported that quantified the reliability of the assay and found that the assay technique developed in our laboratory demonstrates a high degree of reliability. The experiment also provided data on the variability of the degree of branching of glycogen in mice and gave an average value of the degree of branching found in 68 mice. The second experiment sought to determine whether or not a dietary intervention could modulate the activity of the enzyme. Results indicated that mice fed a low carbohydrate diet did not statistically differ from this fed a high carbohydrate diet. Finally, the third experiment analyzed whether or not diet and quercetin supplementation in conjunction would exert an effect on the activity of glycogen branching enzyme. Results of this experiment mainly failed to find statistically significance differences, except in the case of two of the several groups studied. The difference observed between these two groups was difficult to interpret.

One of the points of interest revealed in the experiments is the degree of variability in the degree of branching in mouse liver glycogen. The variance from one animal to the next in terms of both glycogen concentration and degree of branching were both rather high. In liver tissue, this is probably a reasonable expectation. Liver glycogen stores are fairly dynamic in terms of

cycling through synthesis and degradation due to the fact that the liver functions to supply other tissues with glucose in times of need. Depending on the time liver tissue is harvested, whether or not the animal under study had exercised recently, and feeding habits, liver glycogen stores can be expected to vary substantially between individuals. Perhaps it should therefore come as no surprise that the variability of liver glycogen concentration and degree of branching observed in the current study was rather high in relation to the mean values of each reported here.

Skeletal muscle remains an attractive target for investigation of whether or not branching enzyme is modulated whether chronically, acutely, or both. Although Taylor did not report on the method used to assay branching enzyme activity in his 1974 study (*3*), it is of interest that the study reported an increase in branching enzyme activity in response to a chronic exercise in skeletal muscle. It is also important to note that in Pederson's study of transgenic mice overexpressing glycogen synthase the observation of increased glycogen branching enzyme activity took place only in skeletal muscle and not in the liver (*117*). Application of the assay method used in the present study could very well find different results in skeletal muscle than those found in liver. In the present study, the animals were fed *ad libitum* and were not on any exercise regimen. These conditions did not place any metabolic stress on the animals and therefore it would not be expected that the glycogen stores of these animals were depleted. An experimental design that subjected participants to glycogen depleting exercise on a repeated basis or one that examined groups of elite athletes for the degree of branching in skeletal muscle might reveal differences in glycogen structure that can be quantified and compared across multiple studies. Understanding that skeletal muscle glycogen stores are sequestered within the sarcoplasm and not available for export to other tissues, it follows that intramuscular glycogen stores are not as dynamic under normal metabolic conditions and are only mobilized during

metabolic perturbations such as intense exercise or prolonged fasting. We should also expect to find lower variability in skeletal muscle glycogen stores both in terms of glycogen concentration and degree of branching. With this expectation, differences in the degree of branching would be more readily detected. Together with large sample sizes, experiments investigating the degree of branching of skeletal muscle glycogen would be a logical and useful extension of the methods employed in the present study.

The reliability of the assay developed in the course of these experiments should encourage further efforts to apply the assay in new contexts. The cohorts of mice investigated in these experiments were not assigned in a way that was optimized to the purpose of discovering differences in the degree of glycogen branching. The relatively small sample size of each cohort (n=8), limited the power of the study to detect any differences between the groups.

The assay could find fruitful applications in animal models that examine the effect of exercise, disease, and different tissue types. It would be of interest to determine whether or not the degree of branching varies in different muscle fiber types since fast and slow muscle fibers have different metabolic profiles and especially differ in their relative reliance on glycogen as a metabolic fuel. Investigations into the role of exercise in modifying glycogen branching would also add to our understanding of how glycogen structure responds to exercise. Finally, with recognition of the prevalence of diabetes and metabolic syndrome in our society, progress should be made in furthering our understanding of how the structure of glycogen might vary in the liver and muscle tissue of healthy subjects compared to those suffering from metabolic disease. Given the position of glycogen at the nexus of metabolism, the possibility that this readily mobilized source of energy undergoes dysfunctional regulation in disease is one that should not be ignored.

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APPENDIX – DESCRIPTIVE STATISTICS FOR ALL MICE COHORTS

3Week Day Kill LC 50	
Mean	13.20383086
Standard Error	3.596147596
Median	10.30401066
Mode	#N/A
Standard Deviation	10.17144141
Sample Variance	103.4582203
Kurtosis	-1.205700673
Skewness	0.579586826
Range	27.43340268
Minimum	1.114853608
Maximum	28.54825629
Sum	105.6306469
Count	8
Confidence Level(95.0%)	8.503537818

8 Week Night Kill LC 500	
Mean	21.15734579
Standard Error	2.855010036
Median	21.4133556
Mode	#N/A
Standard Deviation	6.993317799
Sample Variance	48.90649384
Kurtosis	-1.437228816
Skewness	-0.933600029
Range	20.16956431
Minimum	9.110185168
Maximum	29.27974948
Sum	126.9440748
Count	6
Confidence Level(95.0%)	7.339036939

8 Week Day Kill LC 50	
Mean	16.75950906
Standard Error	2.994079155
Median	14.94668495
Mode	#N/A
Standard Deviation	8.468534697
Sample Variance	71.71607991
Kurtosis	-0.402652143
Skewness	0.618757133
Range	25.30257195
Minimum	5.452235772
Maximum	30.75480772
Sum	134.0760725
Count	8
Confidence Level(95.0%)	7.079872182

3Week Day Kill HC	
Mean	13.48088686
Standard Error	3.833941034
Median	9.516012994
Mode	#N/A
Standard Deviation	10.84402282
Sample Variance	117.5928308
Kurtosis	-1.996413272
Skewness	0.424503103
Range	26.68680317
Minimum	2.179538323
Maximum	28.86634149
Sum	107.8470949
Count	8
Confidence Level(95.0%)	9.065829948

8W Day Kill LC 500	
Mean	7.267723722
Standard Error	0.950268448
Median	6.715779173
Mode	#N/A
Standard Deviation	2.687765054
Sample Variance	7.224080988
Kurtosis	-0.759563896
Skewness	0.569382741
Range	7.812973683
Minimum	3.942163303
Maximum	11.75513699
Sum	58.14178978
Count	8
Confidence Level(95.0%)	2.247027818

8W Night Kill LC	
Mean	9.713333547
Standard Error	2.821877396
Median	7.680621145
Mode	#N/A
Standard Deviation	7.981474569
Sample Variance	63.70393629
Kurtosis	2.280643328
Skewness	1.435816077
Range	24.59652777
Minimum	1.789537835
Maximum	26.38606561
Sum	77.70666838
Count	8
Confidence Level(95.0%)	6.672679725

3 Week Night Kill HC	
Mean	7.825342582
Standard Error	2.780491853
Median	6.100691017
Mode	#N/A
Standard Deviation	7.356489967
Sample Variance	54.11794463
Kurtosis	5.192031292
Skewness	2.181293298
Range	21.82250066
Minimum	1.95458912
Maximum	23.77708978
Sum	54.77739807
Count	7
Confidence Level(95.0%)	6.803618468

8 Week Day Kill HC	
Mean	14.52005865
Standard Error	3.363701624
Median	13.51917721
Mode	#N/A
Standard Deviation	8.239352626
Sample Variance	67.88693169
Kurtosis	-0.32190902
Skewness	0.244551027
Range	23.22967236
Minimum	3.329174092
Maximum	26.55884645
Sum	87.12035191
Count	6
Confidence Level(95.0%)	8.646670295

8 Week Night Kill HC	
Mean	17.4164672
Standard Error	2.1893893
Median	17.59720538
Mode	#N/A
Standard Deviation	6.192528084
Sample Variance	38.34740407
Kurtosis	-0.852652831
Skewness	-0.354904833
Range	17.47381606
Minimum	7.227827785
Maximum	24.70164385
Sum	139.3317376
Count	8
Confidence Level(95.0%)	5.177083036

VITA

Scott Fuller is originally from Beaumont, Texas but spent his youth in a variety of places including Pittsburgh, Lake Charles, Louisiana, and Taipei, Taiwan, where he graduated from Taipei American School.

After receiving a master of science degree from LSU in Kinesiology in 2005, Scott continued his post-graduate studies and pursued a doctoral degree in Kinesiology with a specialty in exercise physiology. As a graduate assistant in the Kinesiology Department, Scott served as instructor of record for several courses, including Exercise Physiology and Principles of Conditioning. In addition to working in the Kinesiology Department, Scott worked with LSU Athletics, supervising the on-court physical conditioning program for LSU Men's Tennis in the fall of 2008. Scott also served as a consultant to several former LSU tennis players as they transitioned from inter-collegiate to full-time professional competition on the ATP World Tour.

Scott will graduate with a Doctor of Philosophy in Kinesiology with a minor in Biological Sciences in May of 2013.